The granin protein family in cardiac disease

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Abbreviations

HF	heart failure
ACS	acute coronary syndrome
AMI	acute myocardial infarction
LV	left ventricular
NYHA class	New York Heart Association functional class
LVEF	left ventricular ejection fraction
HFPEF	heart failure with preserved ejection fraction
RAAS	renin-angiotensin-aldosterone system
Ca^{2+}	calcium
ECG	electrocardiogram
CT	X-ray computed tomography
cTnT, cTnI	cardiac specific troponin T and I
BNP	B-type natriuretic peptide
NT-proBNP	aminoterminal pro-B-type natriuretic peptide
NSTEMI	non-ST elevation myocardial infarction
PROTECT study	Pro-BNP Outpatient Tailored Chronic Heart Failure Therapy Study
4S	Scandinavian Simvastatin Survival Study
JUPITER	Justification for the Use of Statins in Prevention: an Intervention Trial Evaluating Rosuvastatin
CRP	C-reactive protein
PC	proconvertase
CgA	chromogranin A
CgB	chromogranin B
SgII	secretogranin II
kDa	kilo dalton (unit of mass)
$^{-/-}$	knock out (genetically modified animal)
IP3R	inositol 1,4,5-triphosphate receptor
STEMI	ST elevation myocardial infarction
NF-κβ	nuclear factor kappa-light-chain-enhancer of activated B cells
PRACSIS program	Prognosis and Risk in Acute Coronary Syndromes in Sweden program

GISSI-HF trial	Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto Miocardico- Heart Failure trial
C57BL/6	C57-Black 6 mouse strain
mRNA	messinger RNA
PBS	phosphate buffered saline
DNA	deoxyribonucleotide acids
ELISA	enzyme-linked immunosorbent assay
KHB	Krebs-Henseleit buffer
TTC	triphenyltetrazoliumchloride
RNA	ribonucleotide acids
RT-qPCR	real-time reverse transcription polymerase chain reaction
RIN	RNA integrity number
cDNA	complementary DNA
C_t	threshold cycle
C_q	crossing point
RPL4	ribosomalt protein L4
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
HRP	horseradish peroxidase
CV	coefficient of variation
RIA	radioimmunoassay
TGF-β	transforming growth factor-β
cAMP	cyclic adenosine monophosphate
HIF-1α	hypoxia-inducible factor-1α
LVAD	left ventricular assist device
OPTIMAAL study	Optimal Trial in Myocardial Infarction with Angiotensin II Antagonist Losartan

List of papers in thesis:

Paper #1:

Jansson AM[*], **Røsjø H[*]**, Karlsson T, Hartford M, Flyvbjerg A, Omland T, Caidahl K. Prognostic value of circulating chromogranin A levels in acute coronary syndromes. *Eur Heart J* 2009;30:25-32.

∗ Contributed equally

Paper #2:

Røsjø H, Masson S, Latini R, Flyvbjerg A, Milani V, La Rovere MT, Revera M, Mezzani A, Tognoni G, Tavazzi L, Omland T, on behalf of the GISSI-HF Investigators. Prognostic value of chromogranin A in chronic heart failure. Data from the GISSI-Heart Failure trial. *Eur J Heart Fail* 2010;12:549-56.

Paper #3:

Røsjø H, Husberg C, Dahl MB, Stridsberg M, Sjaastad I, Finsen AV, Carlson CR, Øie E, Omland T, Christensen G. Chromogranin B in heart failure: a putative cardiac biomarker expressed in the failing myocardium. *Circ Heart Failure* 2010;3:503-11.

Paper #4:

Røsjø H, Stridsberg M, Florholmen G, Stensløkken KO, Ottesen AH, Sjaastad I, Husberg C, Dahl MB, Øie E, Louch WE, Omland T, Christensen G. Secretogranin II: a protein increased in the myocardium and circulation in heart failure with cardioprotective properties. *Submitted manuscript.*

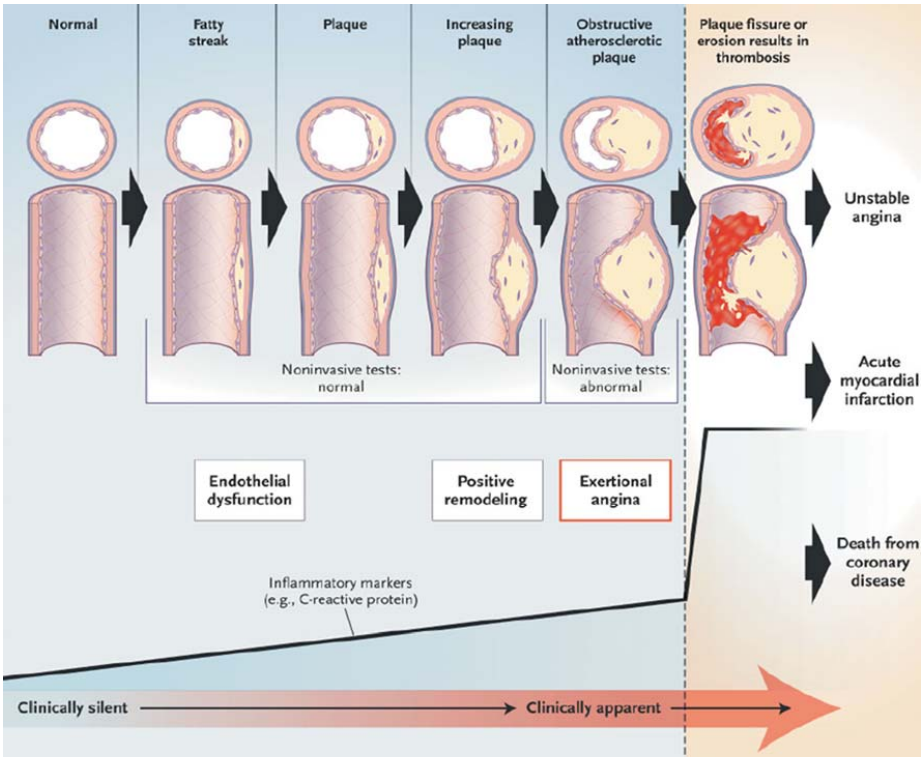
Introduction

Cardiovascular disease

Cardiovascular disease is a leading cause of morbidity and mortality throughout the world.[1] According to the World Health Organisation, 17 million deaths per year can be attributed to cardiovascular disease. This number is expected to rise with the shift in demographics to an aging population in the Western world[2] and an increase in traditional risk factors (smoking, hypertension, diabetes, dyslipidemia) in the developing World.[1] In addition to great human suffering, cardiovascular disease is responsible for substantial economical strain with costs for 2008 alone calculated to $448 Bn in the US and €192 Bn in the EU.[3]

Two principal contributors to mortality in cardiovascular disease are coronary artery disease and heart failure (HF).[1,2,4] Coronary artery disease starts with deposition of lipid particles in the wall of epicardial arteries, which over time progresses to an atherosclerotic plaque (Figure 1).[5,6]

Figure 1. Coronary artery disease represents a continuum from early deposition of lipids in the vessel wall (fatty streak) to the unstable plaque that can rupture and cause a total or subtotal obliteration of the vessel lumen. The latter situation is now classified as an acute coronary syndrome (previously denoted unstable angina pectoris). In stable coronary artery disease, progressive narrowing of the lumen diameter and endothelial dysfunction will result in a mismatch between myocardial oxygen supply and demand that can be experienced by the patient as chest pain (angina pectoris). From Abrams[5] and reproduced by permission from Massachusetts Medical Society. *Copyright Massachusetts Medical Society.*



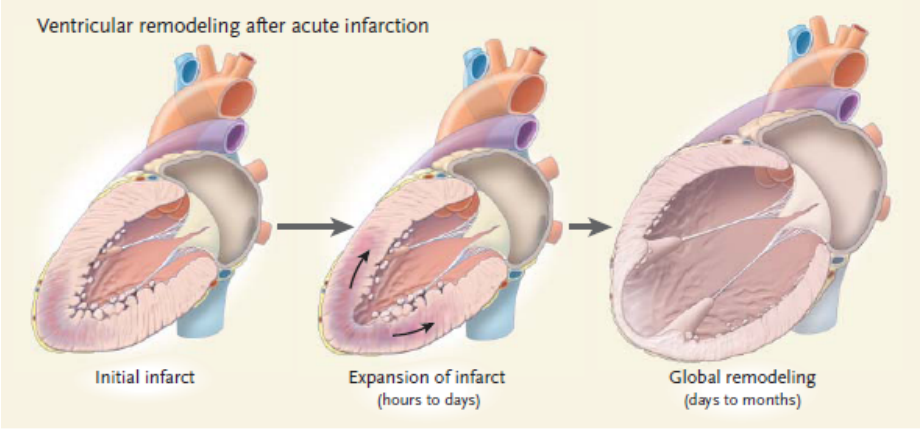
The ratio of fibrosis to lipids determines the stability of the plaque with lipid rich plaques being considered the more unstable lesion.[5,6] The reduction in blood flow of the artery, a consequence of reduced lumen diameter and endothelial dysfunction, results in a mismatch between myocardial oxygen supply and demand.[5] In the classical case, this mismatch is experienced by the patient as chest pain in situations of high myocardial oxygen consumption (angina pectoris).[5] Use of nitroglycerin rapidly normalizes the blood supply by dilating arterial and venous blood vessels, and sublingual nitroglycerin represents an efficient drug for symptomatic relief in patients with angina pectoris.[5] Patients with stable coronary artery disease should also receive treatment with platelet aggregation inhibitors, HMG-CoA reductase inhibitors (statins), and β-blockers to prevent or delay disease progression.[5]

Patients with chest pain and unstable plaques require urgent therapeutic intervention to prevent a total occlusion of the epicardial artery.[7] A model for the unstable plaque relates to the integrity of the luminal cap of the atherosclerotic lesion (Figure 1).[5-7] In this model, unstable plaques are characterized by local inflammation, degradation by matrix metalloproteases,[6] and neovascularization and intraplaque hemorrhages[8] that will destabilize the cap and subsequently result in plaque rupture.[5-8] In the situation of a plaque rupture, molecules in the core of the atherosclerotic plaque will be exposed, platelets will aggregate over the ruptured plaque, and the fibrinogen system will be activated to produce a fibrin clot tethering the platelets to the lesion.[6,7] The triad of plaque rupture, platelet deposition, and fibrin clot will result in total or subtotal obliteration of the vessel lumen and this can happen within seconds (Figure 1). In this setting, which is referred to as an acute coronary syndrome (ACS),[7] nitroglycerin will not alleviate symptoms. If the ACS results in cardiomyocyte cell death, as detected by elevated levels of and a rise-and/or-fall pattern in circulating levels of the cardiac specific troponins, the patient is diagnosed with an acute myocardial infarction (AMI).[7,9] Patients with ACS but no dynamic elevations of troponin levels are diagnosed with unstable angina pectoris.[7,9]

In 2006, coronary artery disease was responsible for approximately 1 out of every 6 US deaths.[10] Improvement in the management of AMI to restore flow in the culprit lesion has reduced the short-term mortality,[11] but late AMI related mortality is still high, often as a consequence of progressive myocardial remodeling.[4] The changes in myocardial structure

after AMI include alterations in both cardiomyocytes and the extracellular matrix,[12,13] which are beneficial in the acute and subacute phase to preserve cardiac output and prevent myocardial rupture. However, in a subgroup of patients these compensatory processes will induce progressive loss of left ventricular (LV) function, and eventually the patient will develop overt HF (Figure 2).[4]

Figure 2. Post-infarction heart failure is a progressive process that starts with the initial infarct, which over days may put a large part of the left ventricle at risk. Within days to months of a large myocardial infarct, global remodeling will induce and this may result in overall ventricular dilatation and decreased systolic function. Local changes in wall pressure and cardiomyocyte stretch together with paracrine and endocrine factors will affect the myocardial cells of both infarcted and non-infarcted myocardial tissue. From Jessup and Brozena[4] and reproduced by permission from Massachusetts Medical Society. *Copyright Massachusetts Medical Society.*



As a consequence of improved treatment of ACS patients the prevalence of elderly subjects with antecedent AMIs will increase in the future.[2,14] The increase in the number of subjects at risk of HF, both patients with prior AMI and elderly subjects with other risk factors for HF[15] will increase the prevalence of HF in the next decade and make HF a key challenge for the cardiovascular community in the 21st century.

The syndrome of heart failure

HF is a syndrome that is categorized into different axes according to etiology, severity, phenotype, and structural pathology.[4] A classical definition of HF is the inability of the heart at normal diastolic pressures or volumes to pump sufficient blood to the peripheral organs,[16,17] which results in a shortage of oxygen and nutrients required for metabolism during rest or activity. Recently, a more clinically oriented definition has been proposed based on the combination of (1) typical HF symptoms (dyspnea, fatigue, ankle edema, etc), (2) typical signs of HF (tachycardia, tachyopnea, pulmonary rales, raised jugular venous pressure, evidence of elevated systemic pressure) and (3) objective evidence of structural or functional abnormality (mainly by echocardiography and raised natriuretic peptide levels, but also cardiomegaly, third heart sound, and cardiac murmurs).[18] There are several conditions, besides AMI, which can result in HF (Table).

Table. Common causes of heart failure

Coronary artery disease/myocardial infarction
Arterial hypertension
Cardiomyopathies
Valvular heart disease
Cardiotoxins/drugs
Congenital heart disease
Tachyarrhythmias
Endocrine diseases: Hypo/hyperthyroidism, pheochromocytoma, etc
Infiltrative diseases: Sarcoidosis, amyloidosis, etc
Infections diseases: Chagas disease, HIV, etc

Establishing the underlying pathology in HF is essential as the etiology will influence therapeutic decisions;[18] e.g. invasive therapy for patients with ischemic etiology, valvular surgery in patients with aortic stenosis, and septal reduction therapy in patients with hypertrophic obstructive cardiomyopathy. The severity of HF also influences the treatment strategy, especially if the intervention is associated with substantial morbidity and mortality (e.g. valvular surgery). Severity of HF is generally classified according to the New York Heart Association (NYHA) functional class system and there is a close association between NYHA class and long-term prognosis.[19] Some therapeutic interventions, e.g. aldosterone blockade and cardiac resynchronization therapy, are currently also only warranted in patients with class III and IV HF.[18]

A third strategy to classify patients with HF, which transcends etiology of HF and disease severity, is to stratify patients according to the left ventricular ejection fraction (LVEF).[4,20] In this system, a cutoff of LVEF=50% divides the patients in two groups: (1) HF with preserved ejection fraction (HFPEF; LVEF≥50%) and (2) HF with systolic dysfunction (LVEF<50%). The principal pathology in patients with HFPEF is impaired relaxation of the myocardium,[21] which leads to inadequate diastolic filling and increased pulmonary and systemic venous pressure (backward failure). In patients with systolic dysfunction, the principal pathology is poorly contracting ventricles resulting in low cardiac output (forward failure),[22] but this may also result in backward failure and elevated pulmonary artery pressure. Hypertension, hypertrophic cardiomyopathy, and aortic valvular stenosis are important etiologies in patients with HFPEF, while systolic dysfunction often is a consequence of antecedent AMI or dilated cardiomyopathy. Of note, some patients with HFPEF can progress to systolic HF and ventricular dilatation with progression of the disease, e.g. in patients with aortic valvular stenosis.

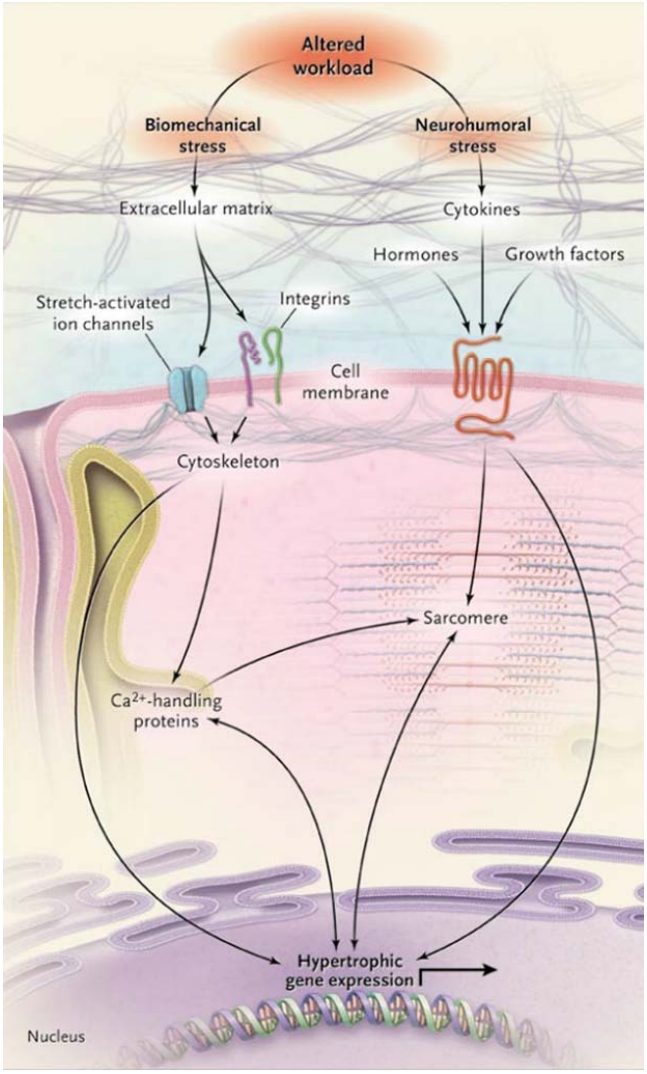
More recently, a fourth strategy to characterize HF has been proposed.[4] This model focuses on the structural alterations of the myocardium and incorporates the progressive nature of HF. The patients are classified in four stages of HF with stage 1 representing the patient at risk of HF (hypertension, diabetes mellitus, etc), stage 2 representing subclinical HF, e.g. structural alterations in the heart but no symptoms, stage 3 overt HF, and stage 4 is end-stage HF requiring transplantation or left ventricular assistant device for survival. This classification provides a conceptual model for studies of HF development, and

emphasizes the need to understand and identify the processes that drive the transition of HF from the patient at risk (stage 1) to the patient in end-stage HF (stage 4). Enhanced knowledge of the key processes in HF progression may improve patient care by identifying new tools for patient management and novel targets for therapeutic intervention.

Basic pathology of heart failure

Progressive loss of myocardial function in HF induces several compensatory mechanisms, both in the myocardium and in extra-cardiac organs.[4,12] Although the etiology of HF influences therapeutic decisions, some pathophysiologic features are considered universal to all HF patients. Principal alterations in the failing myocardium are (1) cardiomyocyte hypertrophy, (2) altered gene expression in myocardial cells, (3) changes in the extracellular matrix composition, (4) alterations in membrane receptors and intracellular signaling, (5) dysfunctional calcium (Ca^{2+}) homeostasis, and (6) enhanced cardiomyocyte apoptosis (Figure 3).[16,23,24]

Figure 3. Key alterations in the myocardium during heart failure development. A single cardiomyocyte is presented together with extracellular matrix. Biomechanical stress and paracrine and endocrine factors will induce alterations in cardiomyocyte membrane receptors and intracellular signaling, modulate calcium handling, and influence gene expression, including initiate hypertrophic gene expression (fetal gene expression). Fibroblast function and the extracellular matrix are also affected in the failing ventricle. From Hill and Olson[23] and reproduced by permission from Massachusetts Medical Society. *Copyright Massachusetts Medical Society.*



These alterations in the failing myocardium seem to be preserved throughout evolution and can also be identified in non-mammalians.[25]

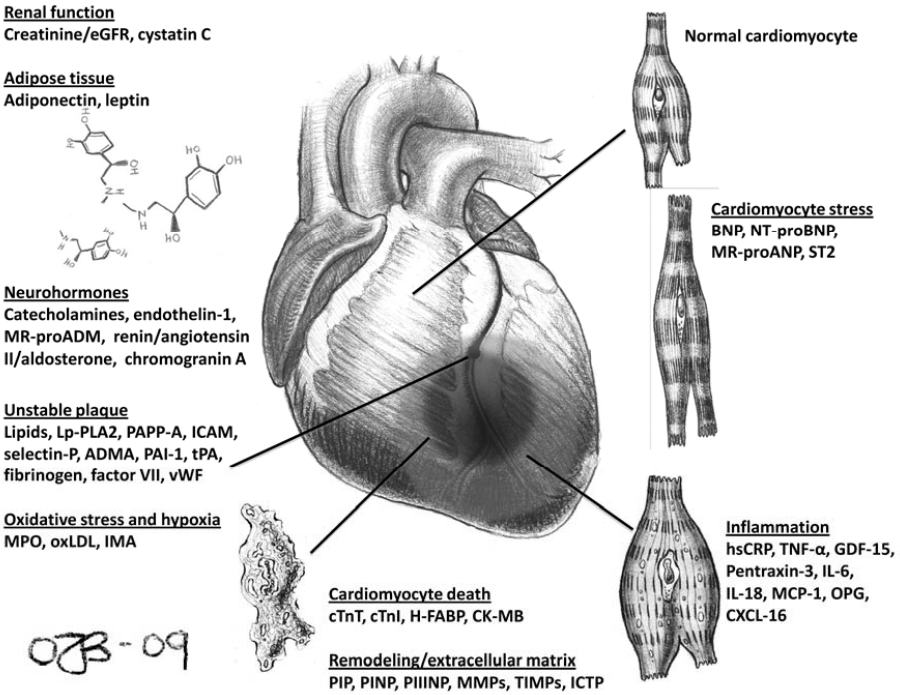
There are also systemic alterations, most prominently increased circulating levels of cathecholamines[26] and the ligands of the renin-angiotensin-aldosterone axis (RAAS)[27] that are universal in HF.[4] Increased neuroendocrine activity is beneficial for short-term survival by maintaining blood pressure, but this comes at the cost of impaired long-term prognosis.[4,12,16,27,28] A prolonged elevation of catecholamine levels and RAAS activity will augment cardiomyocyte oxygen consumption, enhance myocardial fibrosis and hypertrophy, and increase the susceptibility for ventricular arrhythmias by altering intracellular Ca^{2+} handling.[12,16] The importance of catecholamines and the RAAS axis in HF is reflected in contemporary HF therapy where β-adrenergic blockers, angiotensin converting enzyme inhibitors, and angiotensin II receptor blockers all have been shown to reduce HF morbidity and mortality.[18] However, in spite of progress in the treatment of HF, this syndrome still carries a poor prognosis with a dismal 30% five year survival rate recently reported from the Framingham cohort.[29] Moreover, no therapy has so far been proven to efficiently reduce mortality in patients with HF and LVEF>50%.[30] Hence, there is a clear need for better management strategies and therapeutic options in HF. Such novel strategies should explore systemic factors, as well as local alterations in protein synthesis in HF. By providing information on the pathophysiology of HF, these proteins could be of help to guide patient management and possibly also represent novel targets for therapy.

Biomarkers in cardiovascular disease

Diagnosing patients with ACS can be difficult as chest pain is frequent also in non-cardiac conditions such as thoracic myalgias, pulmonary embolism, thoracic abdominal aortic disease, and pleural effusion.[31] Likewise, dyspnea, a cardinal symptom of HF, frequently indicates non-cardiac disease, including asthma and chronic obstructive pulmonary disease, pneumonia, and pulmonary embolism.[32] A late diagnosis of ACS and HF will delay the start of appropriate therapy, and tools that can help improve diagnostic accuracy are therefore of clinical interest.

Biomarkers are defined as a characteristic that is objectively measured and evaluated as an indicator of (normal) physiology, pathophysiology, or as a pharmacological response to therapy.[33] A recording (ECG, Holter, etc) or an imaging test (echocardiogram or CT scan) may be referred to as a biomarker, but the term biomarker is most commonly used in association with measurements of substances in bodily fluids, and especially substances in peripheral blood.[34] Biomarkers are used for diagnosis in several aspects of clinical medicine, including screening for prostate cancer (prostate specific antigen)[35] and to diagnose pregnancy (human chorionic gonadotropin).[36] In the last decades, biomarkers have entered the field of cardiology with great force, although the concept of using circulating markers to diagnose cardiac disease is not new.[37] Prototypical cardiac biomarkers are (1) the cardiac specific troponins I and T (cTnI, cTnT) and (2) peptides derived from pro-B-type natriuretic peptide ($proBNP_{1\text{-}108}$ [subscript indicative of amino acid position]), which are referred to as aminoterminal (NT)-$proBNP_{1\text{-}76}$ and $BNP_{1\text{-}32}$ (e.g. $proBNP_{77\text{-}108}$).[38] Troponins and the BNPs complement each other as troponins is considered to reflect cardiomyocyte necrosis,[39] while proBNP secretion increases by cardiomyocyte stress such as stretch, hypoxia, and inflammation (Figure 4).[40-42]

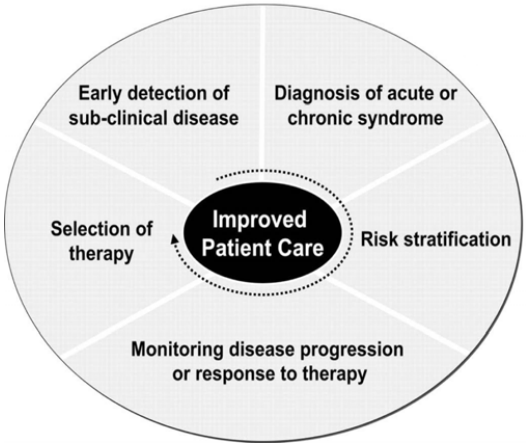
Figure 4. A large number of substances have been proposed as cardiovascular biomarkers. The figure depicts some established and novel biomarkers according to possible pathophysiology reflected by the biomarker, although for the majority of biomarkers this has not been firmly established. The illustration demonstrating an unstable plaque in the left anterior descending artery, myocardial necrosis, and impaired left ventricular function is courtesy of Ole-Jacob Berge, MD.



Of note, the secretion of proBNP is part of the neuroendocrine activation in HF, but represents a counteractive protective mechanism counteracting the effects of the adrenergic system and the RAAS axis.[42]

The organ specificity of troponins and the BNPs makes these ligands excellent biomarkers for identifying cardiac disease with superior sensitivity and specificity to other diagnostic tools.[7,9,18,39] This is also reflected in the current definition of AMI, which is based on a characteristic rise and fall pattern in troponin concentration.[9] Similarly, measurements of BNPs have been shown to complement and improve physician assessment of HF in patients with dyspnea.[32,43] The role of BNPs as diagnostic HF biomarkers is recognized in updated guidelines and elevated levels of the BNPs are included as key factors to identify structural and functional evidence of HF in the most recent guidelines from the European Society of Cardiology.[18] In addition to diagnostic utility, biomarkers are useful for risk stratification, to guide and monitor therapy, and to identify the pathophysiology in the individual patient (phenotype characterization, Figure 5).[44]

Figure 5. Areas in which cardiovascular biomarkers could help improve patient care. From Morrow and de Lemos[44] and reproduced by permission from Wolters Kluwer Health.



For prognosis, both the troponins and BNPs represent strong biomarkers across the spectrum of cardiovascular disease.[45-53] Recently, troponins and natriuretic peptides have also been reported to provide prognostic information in non-cardiac conditions with compromised myocardial function.[54,55] Hence, regardless of the condition, there seems to be a proportional increase in mortality and adverse events with higher levels of troponins and BNPs. Moreover, the information obtained by measuring troponins and BNPs provides incremental information to established risk indices as examined by several statistical methods. A number of other proteins have also been proposed as novel cardiovascular biomarkers (Figure 4),[38] but currently only a minority of these markers has shown real clinical potential and there is a need for further testing before they can be considered for clinical use.[56]

The potential of biomarkers to guide patient management is reflected in the current strategy for non-ST elevation ACS patients.[7] As mortality and adverse events are more common in patients with non-ST elevation AMI (positive troponin profile, NSTEMI) than in patients with normal troponin profile (unstable angina pectoris),[45] patients with NSTEMI are prioritized for angiography over patients with unstable angina pectoris.[7] Similarly, several studies have recently assessed the effect of using BNPs to guide therapy in ambulatory patients with HF.[57-65] The rationale for BNP guided therapy is to identify non-responders and to prevent disease progression at an early stage before the patient develops decompensated HF.[66,67] Although appealing based on the current understanding of proBNP secretion, not all studies with therapy guided by the BNPs have shown superiority over the standard strategy of titrating medication according to guidelines and symptoms.[59,60,62-64] One explanation for the divergent results could be heterogeneity of the patients enrolled in the studies and that several different target of the BNPs have been implemented.[66,67] Furthermore, there seems to be increasing recognition that patients <75 years with systolic HF benefit from BNP guided therapy,[68,69] although also elderly patients profited from NT-proBNP guided therapy in the PROTECT study,[65] which is considered the premium biomarker-guided study so far.[67]

There is increasing recognition among researchers conducting biomarker studies that the field needs to advance from descriptive studies on prognosis to studies that provide more pathophysiological understanding.[67,70] By focusing more on biomarkers that can assist in

subgrouping patients according to pathophysiology; e.g. adrenergic tone, inflammatory status, extracellular matrix turnover, etc., biomarkers may be useful for identifying patient phenotype.[70] This could also help improve our understanding of the complex pathophysiology of HF. To date, a large number of cardiovascular biomarkers have been reported to improve risk assessment;[38] however, few studies have used this information to characterize patient phenotype or to guide therapy.

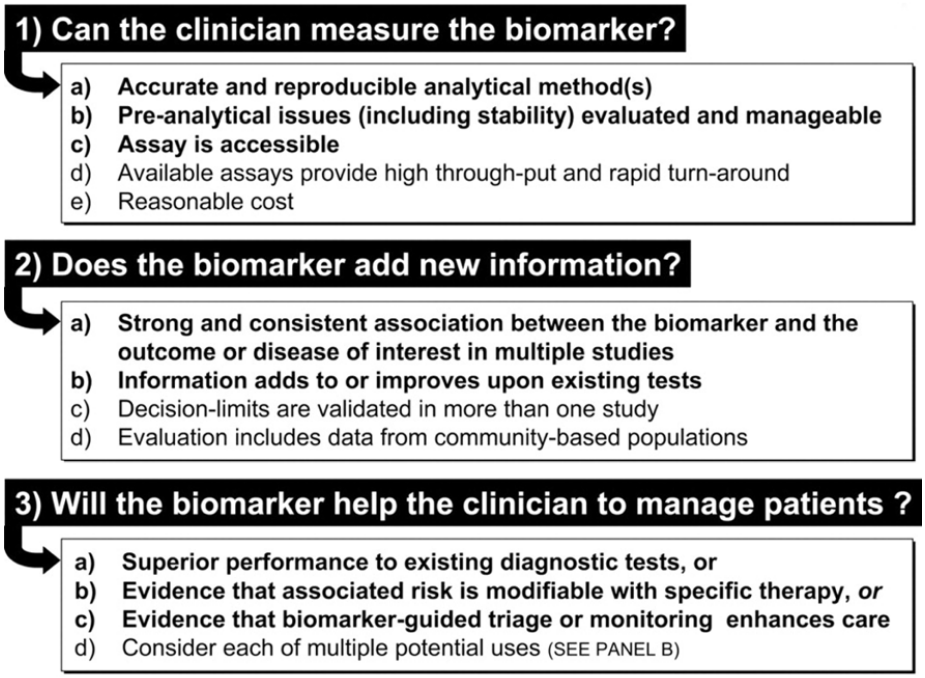
One groundbreaking study that used biomarker levels to include patients was the Scandinavian Simvastatin Survival Study (4S).[71] In 4S, patients with coronary artery disease and total cholesterol levels of 5.5-8.0 mmol/L were randomized to statin therapy or placebo, and there was a consistent reduction in mortality and cardiovascular events in patients on simvastatin. Since then, statin therapy has proved to be beneficial in patients with coronary artery disease in general,[7] but the principle of using lipid levels to identify high-risk patients who will benefit from statin therapy was introduced with 4S. This principle currently forms the basis of recommendations on the use of statins in the general population.[72]

The Justification for the Use of Statins in Prevention: an Intervention Trial Evaluating Rosuvastatin (JUPITER) is another statin study which used biomarkers to guide therapy.[73] In JUPITER, apparently healthy individuals with an acceptable lipid profile, but C-reactive protein (CRP) levels >2.0 mg/L, were randomized to either rosuvastatin or placebo. Analogous to 4S, statin therapy also reduced the risk of the combined primary cardiovascular endpoint in JUPITER, although the numbers needed to treat obviously was much higher in this low risk cohort than in 4S. It is still debated whether the results of JUPITER should be implemented in clinical medicine,[74] but regardless of that, JUPITER was an interesting study by demonstrating that targeting an intervention according to a biomarker cutoff could be successful in a low risk population.

Except for these two statin success stories, limited information is currently available to recommend changes in patient treatment according to biomarker levels. This is also true for the established cardiovascular biomarkers troponins and the BNPs in the non-acute setting, e.g. in patients with subclinical cardiovascular disease or in stable HF. One key factor, which has prevented the use of prognostic biomarkers for guidance of therapy, is

the lack of information on the pathophysiology reflected by most biomarkers. Hopefully, by understanding more of this pathophysiology, physicians will be able to stratify patients according to phenotype and have a rationale for initiating specific therapy in the individual patient (personalized therapy).[70] This strategy contrasts with current guidelines for patients with ACS and HF which recommend that the same therapy should be applied to all patients.[7,18] Still, to utilize the true potential of personalized therapy in ACS and HF, new and established biomarkers should be combined to cover the network of pathophysiology in these complex conditions. The need for additional cardiovascular biomarkers has spurred a search for new cardiovascular biomarkers and several proteins have been proposed as candidates (Figure 4).[38,75] However, to demonstrate clinical potential these biomarkers should fulfill a set of standardized criteria (Figure 6).[44]

Figure 6. Benchmarks for novel cardiovascular biomarkers. From Morrow and de Lemos[44] and reproduced by permission from Wolters Kluwer Health.



According to these benchmarks, the marker should be (1) stable and easy to use, (2) associated with the outcome of interest in several independent cohorts, (3) provide incremental information to standard clinical assessment, and (4) help the physician to treat the patient better.[44] The current statistical methods advocated to explore these new biomarkers are multivariable models to assess incremental information, receiver operating analysis to explore accuracy, and reclassification scores (net reclassification index and integrated discrimination index) to assess calibration of established risk models.[76,77]

A strategy, which seems intriguing but currently lacks sufficient scientific merit, is the use of several markers in a panel (multimarker approach) to provide a comprehensive characterization of the phenotype of the individual patient.[78] The success of the multimarker strategy will depend on the identification of strong new biomarkers, which can complement the information from the established markers troponins and the BNPs.

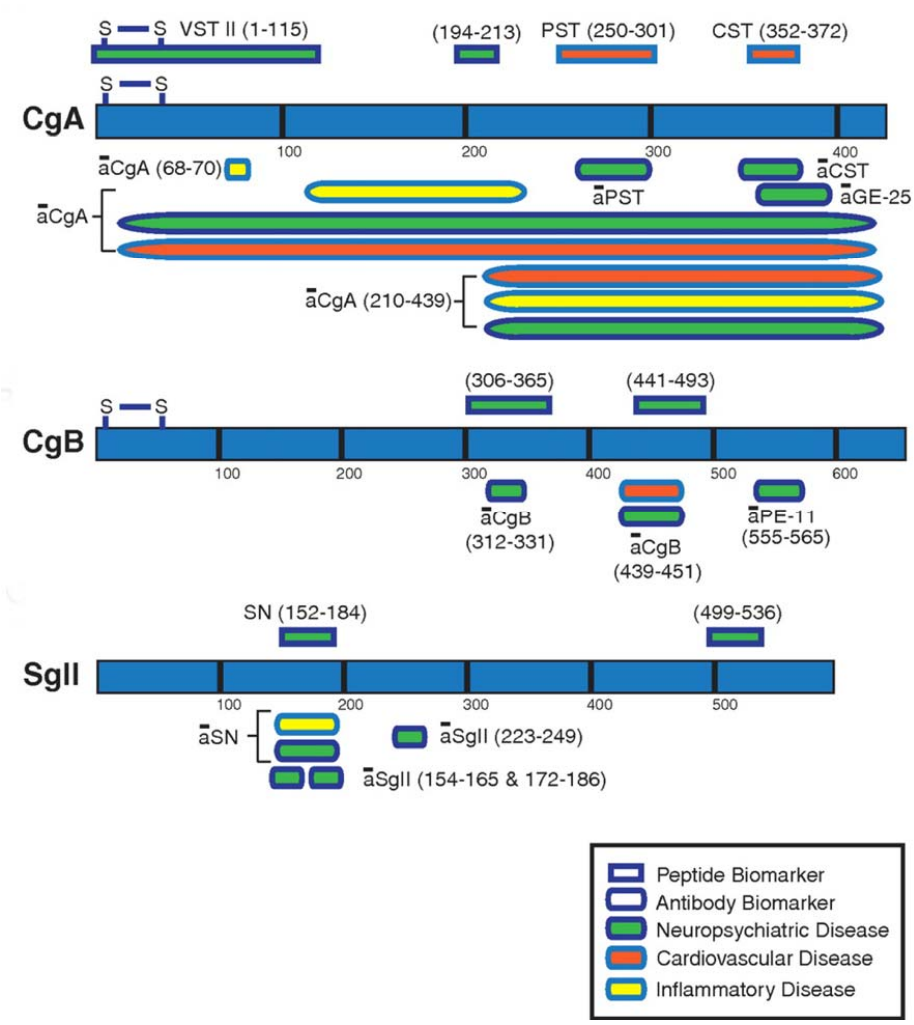
Production of granin proteins

The chromogranin-secretogranin (granin) proteins are a family of proteins characterized by a high proportion of acidic amino acids and several dibasic cleavage sites.[79] Post-translational processing of the granin proteins at cleavage sites produces multiple short ~30 amino acid peptides.[79-82] This extensive processing of the precursor prohormone is a hallmark of the granin protein family.[79] Proconvertase (PC) 1/3 and PC2 constitute the most important proteases involved in the processing of the granin proteins,[83,84] although plasmin has also been identified as an enzyme which can cleave chromogranin (Cg) A.[85]
The granin proteins have several characteristics in common with other prohormones, such as proopiomelanocortin and proenkephalin, but can be differentiated from the other prohormones by the large size of the granin proteins, which is >400 amino acids for CgA, CgB, and secretogranin (Sg) II.[79] The calculated molecular mass of CgA, CgB, and SgII are ≥50 kDa, while the largest classical neuropeptide prohormones have a size of approximately 30 kDa (proopiomelanocortin and proenkephalin of ~265 amino acids).[79] Furthermore, most other prohormones are only processed at one cleavage site producing two shorter fragments, while 9-16 cleavage sites have been reported for CgA, CgB and SgII.[79] The high number of cleavage sites result in complex processing of the prohormones with multiple shorter granin peptides[79,80] and this was identified already

from the early studies on granin processing.[86-88] The processing of granin proteins is also less stringent compared to other prohormones,[79] which results in partially processed truncated peptide fragments together with some fully processed peptides. The complexity of granin processing makes the characterization of the short peptides more complicated than for most other prohormones.[79] Still, heterogeneity of processed proteins is not unique to the granin proteins and this has also been demonstrated for $proBNP_{1-108}$ processing[89] with a substantial proportion of circulating BNP_{1-32} being truncated fragments.[90]

The index member of the granins, CgA, is a 439 amino protein (Figure 7) which was first demonstrated in the adrenal medulla,[91] but later has been identified throughout the neuroendocrine system.[92]

Figure 7. Overview of the principal granin proteins chromogranin (Cg) A and B and secretogranin (Sg) II. Shorter fragments of the granin proteins are also presented together with the proposed role of these peptides in pathophysiology and as disease biomarkers (not including data from paper #4 of this thesis). Vasostatin II is presented as VST $II_{1\text{-}115}$ and catestin as $CST_{352\text{-}372}$ under CgA, while secretoneurin is presented as $SN_{154\text{-}184}$ under SgII. From Bartolomucci et al[79] and reproduced by permission from the Endocrine Society. *Copyright 2011, The Endocrine Society.*



The synthesis of CgA in the neuroendocrine system, and the increased production in malignant cells,[93] has made circulating CgA a clinical useful marker of neuroendocrine tumors (pheochromocytomas, carcinoids, etc.).[94] Moreover, circulating levels of CgA are closely correlated with catecholamine levels in situations of enhanced adrenergic tone,[95-97] hence promoting a role for CgA as an index of neuroendocrine activity during stress.[98] This could be of clinical value as technical requirements for catecholamine analysis renders epinephrine and norepinephrine measurements impractical for routine use, while CgA seems to be a stable protein with a high signal-to-noise ratio[99] that can be analyzed in a general clinical laboratory.[100] Still, it should be acknowledged that circulating CgA levels are not a perfect surrogate marker for adrenergic tone as CgA is also produced in non-neuroendocrine cells.[101-106] Moreover, the production in non-neuroendocrine organs seems to increase with pathology,[105-109] which suggests that these organs could make a more pronounced contribution to circulating levels during disease than in the steady-state situation. This could be relevant for cardiovascular disease as cardiomyocytes increase their CgA synthesis in HF.[109]

CgB and SgII are the two other principal granin proteins. CgB is a 657 amino acid protein with an N-terminal loop due to a disulfide bridge which is also present in CgA (Figure 7).[79] This characteristic 3-D structure forms the basis for the classification of these proteins as chromogranins, while SgII is a 587 amino acid protein with a linear aminoterminal end (Figure 7).[79]

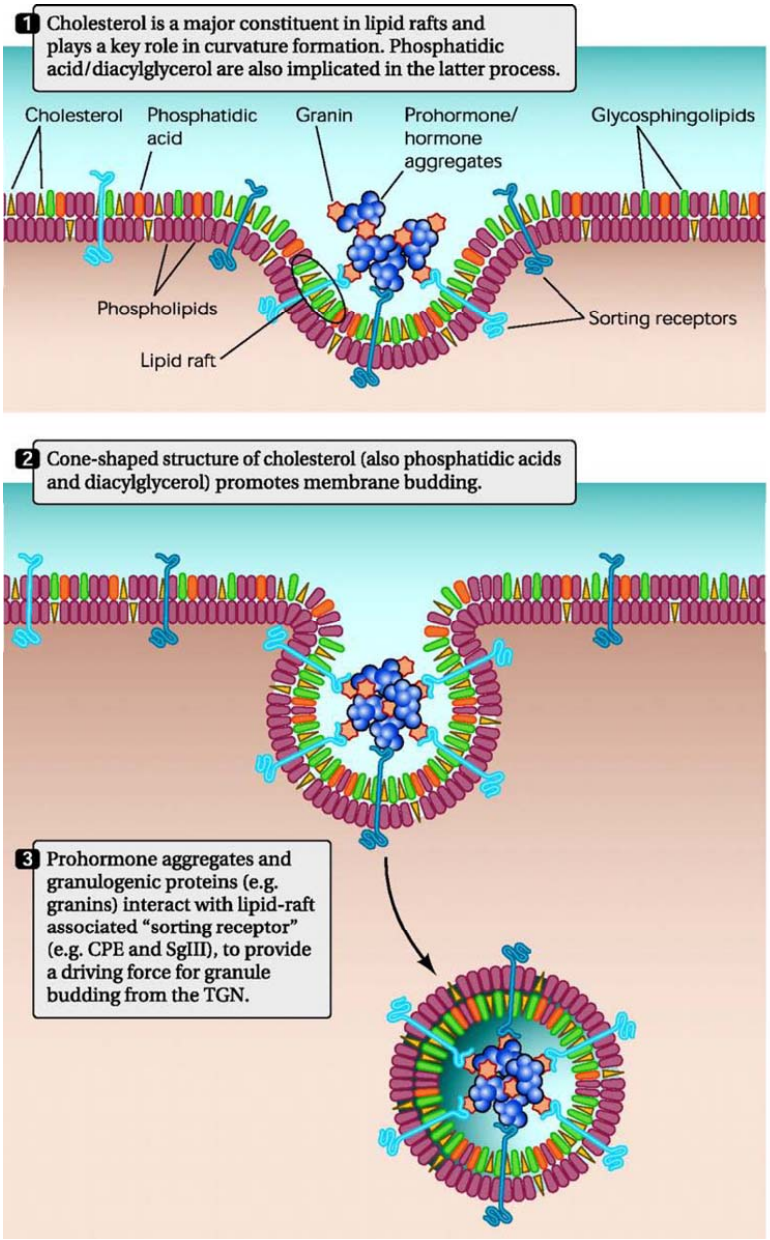
CgB and SgII are also produced throughout the neuroendocrine system,[88] (chromogranin B is denoted as secretogranin I in this paper) but the expression of the granin proteins is not uniform across organs[110,111] with high chromogranin concentration in the adrenal medulla,[86,112] while SgII production is prominent in the pituitary gland.[112,113] Analogous to the results for CgA, there are also organs outside of the neuroendocrine system that produce CgB and SgII.[102,108] The ratio between production in the neuroendocrine system and non-neuroendocrine organs has not established for these granins and may also be different for CgB and SgII.

Regardless of the localization, a common denominator for cells that produce granins seems to be a high secretory rate.[86-88,92-94,101-103,107-113] In contrast to the previous outdated model of granin production in the neuroendocrine system,[80,81] the literature now suggests that all cells with marked exocytosis will have granin production. This new model for chromogranin A production is supported by enhanced synthesis of granin proteins in cells that are transformed to a secretory phenotype during later stages of disease. Prominent examples of such cells are tumor cells, e.g. in small cell lung cancers[104,105] and in prostate cancers,[108,114] and cardiomyocytes during HF development.[109] Whether this model was relevant also for CgB and SgII production was not established prior to the work described herein.

Physiological role of granin proteins

The model of granin production in cells with marked exocytosis is supported by the importance of the granin proteins for granulogenesis.[115-119] Granin proteins stabilize intracellular granula by binding Ca^{2+} and proteins, and the granin proteins are key elements in the nucleus of secretory vesicles destined for regulated secretion (Figure 8).[120]

Figure 8. The granin proteins are crucial for granulogenesis. The granin proteins stabilize granula by binding to lipid-raft sorting receptors and by forming a dense core substrate with calcium and prohormones and peptides destined for regulated exocytosis. From Kim et al[120] and reproduced by permission from the American Physiological Society.



The prominence of granin proteins for granulogenesis has been convincingly shown *in vitro* and *in vivo*, including in mouse CgA and CgB knock-out ($CgA^{-/-}$ and $CgB^{-/-}$) models.[121-123] In the $CgA^{-/-}$ mice, catecholamine levels were elevated due to impaired granulogenesis and depletion of secretory vesicles in the adrenal medulla.[121] Of note, these alterations were obliterated after transfecting the $CgA^{-/-}$ mice with the human variant of the CgA gene. Analogous, $CgB^{-/-}$ mice demonstrated alterations in the release of insulin, glucagon and somatostatin from the pancreas.[123] The effect of CgB for granulogenesis has also been demonstrated *in vitro* by overexpressing CgB in non-neuroendocrine cells.[124] The role of SgII in granulogenesis has not been established, but we postulate that also SgII will influence granula stability as this protein co-localize with the chromogranins and binds Ca^{2+} with superior affinity to CgA.[125]

The ability of granin proteins to bind Ca^{2+} could also be important for intracellular processes besides granulogenesis.[126] Granin proteins interact with the luminal side of the inositol 1,4,5-triphosphate receptor (IP3R),[127,128] a receptor of increasing importance in cardiac disease,[129] and enhance the Ca^{2+} efflux through the receptor complex.[125,126,128,130-132] Among the granins, CgB has superior affinity for Ca^{2+} binding and also provides the strongest influence on IP3R activity.[128] The combination of high-capacity, low-affinity Ca^{2+} binding and a positive effect on receptor activity[126] indicate a role for granin proteins in cardiomyocyte Ca^{2+} homeostasis. Of note, this model was recently supported for CgB in the myocardium where CgB was an intermediate in angiotensin II-induced Ca^{2+} signaling and cardiomyocyte hypertrophy.[133] Whether CgB may modulate cardiomyocyte Ca^{2+} channels besides the IP3R has not been examined, and currently no information is available on the association between CgA and SgII and Ca^{2+} channels in the myocardium.

The extracellular properties of the granin proteins are attributed to the effects of the multiple short ~30 amino acids peptide fragments.[79,80] However, despite the high rate of processing and the high number of fragments, only a minority of these peptides have been found to influence cellular function. Extrapolating from experimental animal models, the two CgA fragments vasostatin II (CgA_{1-113}) and catestatin ($CgA_{344-364}$) seem especially important for cardiovascular pathophysiology.[134] A peptide from SgII, secretoneurin ($SgII_{154-186}$), could also have functional properties of relevance for cardiovascular disease.[135,136] However, prior to this work, no study had assessed the functional relevance

of SgII in cardiovascular disease. In addition, other granin fragments have been identified, including pancreastatin ($CgA_{240-288}$) that influences glucose metabolism and CgB fragments of the innate immune system,[79,80] but currently these peptides have not been assigned a role in ACS or HF.

The CgA fragment vasostatin II influences cardiovascular physiology by directly promoting peripheral vasodilatation[137] and by counteracting β-adrenergic stimulation of contractility[109,138] and excessive β-adrenergic and endothelin-1 signaling in cardiomyocytes.[139] Vasostatin II has also been found to prevent tumor necrosis factor-α enhancement in endothelial cell permeability[140] by modulating the endothelial cytoskeleton,[141] which could be important in HF to reduce symptoms of congestion and to improve prognosis. A negative inotropic effect has also been demonstrated for catestatin.[142] Moreover, as catestatin reduces secretion of catecholamines from the adrenal medulla[143] and modulates β-adrenergic signaling in cardiomyocytes,[144] catestatin could represent an important counteractive mechanism to excessive adrenergic drive in patients with cardiovascular disease.[145,146] Catestatin has also been demonstrated to protect from myocardial ischemia-reperfusion injury in the Langendorff model[147] and to induce angiogenesis *in vitro* and *in vivo*.[148]

The protection by CgA and catestatin to catecholamines could be of relevance for essential hypertension as studies have found specific genetic alterations linked to CgA[149,150] and catestatin synthesis[151] associated with elevated blood pressure. The promoter region of CgA controls the gene expression by mediating responsiveness to transcription factors and variants (polymorphism) in this region will influence CgA synthesis.[152] In contrast, direct alterations in the CgA gene will influence the amino acid sequence of the protein and render the protein more susceptible for degradation or alter the potency of the protein by changing the 3-D structure.[142] The association between CgA production and hypertension is supported by a twin-heritage linkage study in which the subjects at risk of hypertension had reduced circulating catestatin levels prior to developing hypertension.[151]

A third peptide from the granin protein family which could play a role in cardiovascular pathophysiology is the 33 amino acid peptide secretoneurin.[136] This peptide has been found to attenuate ischemia-reperfusion injury in skeletal muscle[153] and the brain,[154] but

an effect of secretoneurin on myocardial injury has not been examined. Secretoneurin also protects against apoptosis and potently induces angiogenesis[155] and vasculogenesis,[156] processes that could be important for large groups of patients with cardiovascular disease.

Conceptual model for granin proteins in cardiac disease

The model of the heart as an endocrine organ was postulated by Braunwald in 1964 in relation to norepinephrine production in the myocardium.[157] This model was advanced by de Bold and co-workers in 1989 with the discovery of atrial natriuretic peptide[158] and by the group of Nakao and Imura in 1991 that identified $proBNP_{1-108}$ as the principal hormone of the ventricles.[159] Analogous to the phenotype of several malignancies, the cardiomyocytes attain a secretory phenotype with advanced stages of HF. The close association between myocardial function and secretion of $proBNP_{1-108}$[159] forms the basis for the sensitivity and specificity of BNP and NT-proBNP as cardiac biomarkers.[42] The association between myocardial function and cardiomyocyte protein secretion also provides the rationale for examining other myocardium synthesized proteins as novel cardiovascular biomarkers.

The model of the heart as an endocrine organ formed the basis for the first study on the granin proteins in cardiovascular pathophysiology.[101] The investigators hypothesized that CgA and CgB would be co-localized with natriuretic peptides in the atrial granula, which was similar to the role of the chromogranins as the core substrate in secretory granula of neuroendocrine cells. Supporting their hypothesis, the authors were able to demonstrate CgA and CgB production in atrial cardiomyocytes and to show co-localization between atrial natriuretic peptide and chromogranins in granula.[101] CgA was also found to be processed to shorter fragments in atrial tissue, while there was no post-translational CgB processing. A comparison between chromogranin production and processing in the healthy and the diseased myocardium was not performed.

A different avenue of granin research in the early 1990s related to the association between circulating CgA and catecholamine levels. Enhanced activity in the neuroendocrine system and elevated levels of catecholamines are acknowledged risk factors in cardiac disease,[28] but epinephrine and norepinephrine play a modest role as clinical biomarkers

due to the short half-life and large day-to-day and time-of-day variation of these hormones.[98] Analysis of catecholamines are also time consuming and requires complicated analytical methods, most often high-performance liquid chromatography. In contrast, both CgA and CgB have been reported to be robust and stable proteins with a high signal-to-noise ratio.[99,160] Accordingly, CgA was tested as a surrogate marker for catecholamines, and this association was validated in situations of marked neuroendocrine activity with correlation coefficients of 0.7-0.8.[95-97] In contrast, there was only modest correlation at rest and CgA levels seem to represent a valuable index of neuroendocrine activity in situations of marked stress, but not in the steady-state situation.

The importance of CgA for adrenal medulla function and catecholamine secretion was later also supported by studies performed by the group of O'Connor using the genetically modified $CgA^{-/-}$ mouse.[121] The CgA knock-out mice showed marked depletion of granula in the adrenal medulla, but elevated circulating catecholamine levels. The higher levels of circulating catecholamines were attributed to defective storage of catecholamines in the adrenal medulla and subsequent constitutive catecholamine release. Moreover, these mice exhibited evidence of LV hypertrophy and impaired diastolic and systolic LV function, which were prevented by inserting the human variant of the CgA gene in the in $CgA^{-/-}$ mice. The myocardial remodeling in $CgA^{-/-}$ mice was also prevented by the infusion of the CgA fragment catestatin.[121]

The merit of CgA as an index of neuroendocrine activity was cited by the group of Ferrari as the rationale for testing CgA as a cardiovascular biomarker.[161] In a group of 160 patients with chronic HF, Ceconi et al found CgA levels to be increased in proportion to HF severity as measured by the NYHA functional class. Moreover, CgA levels were strong predictors of mortality or the need for cardiac transplantation. The association between CgA levels and the endpoint of mortality or transplantation was also statistically significant in multivariable analysis, including a model that adjusted for LVEF, NYHA class, and BNP and norepinephrine levels. Omland and coworkers have later demonstrated prognostic utility of CgA measurements in two cohorts of patient with ST elevation myocardial infarction (STEMI).[162,163] Of note, in the two latter studies there were no significant correlations between circulating CgA and catecholamine levels. This was surprising based on the literature and could indicate additional organs besides the

neuroendocrine system contributing to circulating CgA levels in cardiovascular disease.[164] However, there was heterogeneous blood sampling (range day 1-10) in the STEMI studies[162,163] and the data should not be interpreted as evidence that the adrenal medulla does not contribute to circulating CgA levels in ACS or HF patients. Other studies of patients with more extreme sympathetic activation have demonstrated a close correlation between CgA and norepinephrine levels, including during cardiac arrest and resuscitation ($r=0.84$, $p<0.001$).[96]

Finally, in the initial stages of this work, an Italian consortium reported increased production of CgA in the failing myocardium, including N-terminal CgA fragments.[109] This could be of importance as vasostatin II has been found to counteract β-adrenergic inotropic effects in experimental models.[134] Moreover, CgA was found to co-localize with $proBNP_{1-108}$ in cardiomyocytes. The presence of N-terminal CgA fragments in the heart has later been verified by a proteomics approach in the healthy rat myocardium.[165]

Another recently published paper found CgB to be a positive modulator of IP3R activity in cardiomyocytes *in vitro* and *in vivo*.[133] In this work, CgB was also identified as an important intermediate in cardiomyocyte angiotensin II signaling by regulating IP3R-mediated Ca^{2+} efflux from the sarcoplasmic reticulum. The results of this study support a model in which CgB, IP3R, and the transcription factor NF-κβ regulate myocardial hypertrophy and influence $proBNP_{1-108}$ production, thus linking CgB expression directly to the synthesis of BNP and NT-proBNP in the LV. This work also found CgB levels to be increased in the hypertrophic LV after angiotensin II infusion, but the investigators did not examine CgB production in HF models. Thus, although some information were available prior to the start of this project in 2006,[164,166] there were several unanswered questions relating to the chromogranins in cardiovascular disease that we wanted to explore in this thesis. Moreover, no information was available on the role SgII plays in cardiovascular disease.

Aim of thesis

The general aim of this thesis is to examine the role granin proteins play in cardiovascular disease

Specific aims:

1st paper: To examine CgA as a prognostic biomarker across the spectrum of ACS, e.g. in patients with STEMI, NSTEMI, and unstable angina pectoris

2nd paper: To examine CgA as a prognostic biomarker in a large cohort of stable HF patients treated according to updated guidelines

3rd paper: To examine CgB production and circulating levels in HF by experimental and clinical studies

4th paper: To examine SgII levels in HF and effects of the SgII fragment secretoneurin on post-infarction HF development by a translational investigational program

Methodological considerations

Clinical biomarker studies

There are four different clinical cohorts included in this thesis: (1) 1268 patients from the Prognosis and Risk in Acute Coronary Syndromes in Sweden (PRACSIS) program included during mid-September 1995 to mid-March 2001 at Sahlgrenska University Hospital, Gothenburg, Sweden, (2) 1233 patients included from Aug 6 2002 to Feb 28 2005 in the GISSI-HF trial of Italy and Switzerland, (3) 80 patients recruited during 2007 at Akershus University Hospital, Lørenskog, Norway: 70 patients from the outpatient HF clinic and 10 patients hospitalized for acute HF, and (4) 58 ambulatory patients with stable HF (no hospitalization for acute HF <3 months prior to study commencement) from cohort #3.

The first two cohorts are prespecified biomarker substudies of the large clinical studies PRACSIS[167] and GISSI-HF.[168] In the PRACSIS program, 2335 patients were included to study the epidemiology of ACS in Sweden. In the GISSI-HF trial, 6975 patients were included to assess the effect of add-on treatment with rosuvastatin[169] and n-3 polyunsaturated fatty acids[170] to conventional therapy in stable HF. The selection of only a subgroup of the patients to the biomarker studies might reduce the external validity of the results. This may be partially correct for the PRACSIS biomarker study, in which a higher proportion of patients were diagnosed with AMI compared to the prevalence of AMI in the total PRACSIS cohort.[171] For the GISSI-HF study, there were no differences in baseline characteristics between the patients in the GISSI-HF biomarker substudy and the rest of the GISSI-HF trial.[172] The large sample size (>1000 patients) in papers #1 and #2 support that our results are valid in the majority of patients with ACS and stable HF.

The two last cohorts were recruited from Akershus University Hospital, a teaching hospital situated in the greater Oslo region with a catchment area of 460000. In paper #3, we recruited 70 patients from the outpatient HF clinic while the last 10 patients were recruited among patients hospitalized for acute-on-chronic HF at Akershus University Hospital.[173] For paper #4, only the patients with stable HF were included from cohort #3 to obtain a more homogenous cohort.[174] Stable HF was defined as no hospitalization for

acute HF during the last 3 months prior to study commencement. All of the clinical studies were conducted according to the Declaration of Helsinki, approved by the Regional Ethics Committees, and all participants signed an informed consent prior to study commencement.

The clinical relevance of a biomarker is characterized by several features, but a core fundamental is the ability of the biomarker to improve established prognostic, diagnostic, or therapeutic strategies.[44] Accordingly, to enable assessment of incremental information, diligent information on patient history, clinical examination, and the results of diagnostic and prognostic tests should be collected. The clinical cohorts of this thesis were all prospectively planned and all patients were subjected to an extensive clinical examination. All patients in cohorts #2-4 have measurements of BNPs and most patients also had an updated echocardiographic examination. In cohort #1, standard clinical and laboratory data from the hospital admission were available in all patients, while cTnT and proBNP measurements were only available in a subgroup of patients as these biomarkers were not part of the clinical routine in the first years of the PRACSIS study. A large proportion of the patients in PRACSIS have echocardiographic measurement of LVEF, which is a strength of the study.

We assessed incremental information by CgA to established risk indices in papers #1 and #2 by multivariable logistic regression analysis. In the GISSI-HF substudy, we also calculated the *C* statistic of established risk factors and compared this to the *C* statistic of established risk factors combined with CgA measurements. Receiver operating characteristics analysis was not performed in paper #1.

Experimental HF model

The use of experimental animal models has been instrumental in promoting cardiovascular research in the 20th century.[175] The ability of the researcher to control all aspects of the experiment provides a unique model in which external confounders can be largely controlled for. In contrast, factors such as genetic make-up, age, gender, comorbidity, and medication will influence results in patients, thus reducing the ability to make definite assumptions of causality in clinical studies.

Until the late 1970s, basic cardiac research was performed in large animal models such as pig, dog or sheep.[176] The strengths of these models are the size of the animal, which makes surgery more easy, and the possibility to perform serial blood sampling without compromising hemodynamic stability. However, as large animal models are expensive and require large animal facilities, these models have now largely been replaced by small animal models.[175] The progress in microsurgery has permitted most experimental models originally developed in large animals to be transferred to mice and rats. This also relates to the post-AMI HF rodent model with a permanent ligation of the left main coronary artery, which was refined by Pfeffer and coworkers in the late 1970s.[177] By using mice and rat the influence by genetic variation is also annihilated due to special breeding strategies, which produce animals with identical genetic make-up.[178] The stringent control of microbiological contamination in rodent breeding facilities ensures that all animals are supplied in good health, which will reduce variation due to non-controlled external factors. Accordingly, the many advantages of mice and rats over larger animal models have changed experimental cardiac research during the last three decades. Of note, although obviously different by phenotype, examination of the mouse genome has demonstrated that 99% of mouse genes have a human homolog, which supports the validity of rodent models to explore human disease.[179]

We have used the mouse strain C57-Black 6 (C57BL/6) for our *in vivo* work in paper #3 and #4. The C57BL/6 strain has nearly identical genetic make-up due to several generations of inbreeding and is widely used in cardiovascular research. The animals were obtained in week 5 and acclimatized for one week in-house before surgery was performed in week 6. Only male mice were used to avoid influence by the cyclic variation of sex hormone. To reduce the effect of the anesthesia on cardiac function, animals were anesthetized with propofol and isoflurane, which have less cardiodepressive effects than other anesthetics. After being trachetomized, the mice were connected to an animal ventilator and ventilated with a mixture of 98% oxygen and 2% isoflurane throughout the surgery. Via a left-sided thoracotomy, pericardectomy was performed followed by ligation of the left main coronary artery in the HF group. After finishing surgery, all animals received 0.01 ml buprenorfin s.c. before being weaned from the ventilator.

As the surgery alone can induce changes to the animals, it is important to compare animals in the HF group to control animals that also have been subjected to anesthesia and surgical trauma. We obtained relevant control animals by performing sham surgery on animals that were also trachetomized, mechanically ventilated, and subjected to thoracotomy and pericardectomy but had no coronary artery ligation. By comparing granin levels in HF animals with sham animals, the increase in granin synthesis was not a result of surgery *per se*, but rather supports our hypothesis of enhanced granin production in HF.

Although widely used, some limitations to the post-infarction HF mouse model should be acknowledged. The most striking difference relates to the healthy endothelium of the coronary arteries in mice compared to the widespread atherosclerosis found in elderly patients with coronary artery disease and HF.[5] However, as collateral circulation seems to protect in the setting of acute ischemia,[180] this could actually represent a disadvantage for the animal model. Another difference is the use of young mice for our experiments while the median age for AMI is 67 years in men and 70 years in women.[181] As aging influences myocardial structure and function,[182-184] our model could overestimate the plasticity of the myocardium to recover after AMI. Still, our goal was not to assess morbidity and mortality after AMI, but rather to explore the hypothesis of enhanced granin production in the failing myocardium, and we believe our model is valid to answer this question.

Strict echocardiographic criteria, which previously have been validated by invasive pressure measurements,[185] were employed to make sure that only animals in decompensated systolic HF were included in the HF group. The presence of myocardial remodeling and congestion were confirmed by measuring LV mass and lung weight in all animals. Other groups have previously also demonstrated substantial alterations in gene expression one week after AMI in mice with HF,[186] which indicates that our model recaptures key molecular alterations in patients with HF. Accordingly, the model and the time point we chose to study granin expression in HF should give us representative data which can be extrapolated to post-AMI HF development in patients.

All of the animal experiments were performed according to established guidelines and were approved by the Norwegian Animal Research Authority.

Echocardiography

Echocardiography is the principal method to assess myocardial structure and function. The estimation of LVEF represents the most common index by which to quantify systolic function.[187] In this thesis, we report LVEF in all patients with HF (papers #II-IV), although not all patients had a recent examination as they were deemed stable by the treating physician and thus not in need of a new echocardiography. LVEF was also calculated in the majority of patients in paper #I (990 of the 1268 patients, 78%), which is superior to the proportion of patients with LVEF measurements in other ACS biomarker studies.[49,52,188-190]

Echocardiography was also used to identify the mice with systolic HF one week after AMI. The tachycardia of the mice and the short distance from the thoracic wall to the heart represent technical challenges for echocardiography,[191] but this can be compensated for by proper equipment and an experienced investigator. For all our echocardiographic examinations in mice, we used a special small rodent transducer. This system produces high quality images and helped us differentiate between animals with HF and animals with minor infarcts and preserved systolic function. To only include animals that were in HF, we used echocardiographic criteria that are considered sensitive and specific for HF and which previously have been validated against invasive hemodynamic measurements: (1) AMI >40% of the circumference of the LV and (2) left atrial diameter >2.0 mm.[185] Increased lung weight was also an absolute criterion for inclusion in the HF group to ensure that only animals with congestion were included in the HF group.

The effect of anesthesia on cardiac function was reduced to a minimum by using oxygen and isoflurane supplied via a facemask during the echocardiography.

Cardiomyocyte cell culture experiments

The use of isolated cardiomyocytes in basic cardiovascular research dates back to 1912 when Burrows first demonstrated that cardiomyocytes could contract *in vitro*.[192] Cardiomyocytes respond in a similar fashion to external stimuli *in vitro* as they do *in vivo*, thus making this model relevant for studies of cardiovascular pathophysiology.[193] Neonatal cardiomyocytes, which are not terminally differentiated cells, represent an optimal model to assess alterations in growth and gene expression as they show dynamic changes to external stimuli and can activate fetal gene expression,[194,195] which is a key feature of the failing myocardium.[196] Analogous to other experimental models, a major advantage of using isolated cardiomyocytes is the ability of the researcher to control external confounders.

We used neonatal rat (Wistar) cardiomyocytes in papers #III and IV to assess the influence by hormones, cytokines, and growth factors on granin gene expression. The ligands that were included in our panel are all increased in HF and have previously been shown to influence cardiomyocyte gene expression.[193] In paper #4, neonatal rat cardiomyocytes were also used to explore the effect of the SgII fragment secretoneurin on hydrogen peroxide-induced cardiomyocyte apoptosis and on protective intracellular signaling pathways.

To obtain pure cultures of neonatal cardiomyocytes, the cell suspension was filtrated through a discontinuous Percol gradient after enzymatic digestion.[197] During the enzymatic digestion of the cells, the cell membrane may be damaged and a recovery period is therefore recommended to allow repair of superficial membrane damage and to eliminate lethally damaged cells.[198] In our experiments, all cells were allowed to recover for 24 h prior to the start of experiments. We also visually inspected the cardiomyocytes to assess the number and quality of cells. Moreover, positive control ligands were included in all experiments to assure that the cells responded in a uniform manner throughout the series of experiments. For the studies on granin messenger (m)RNA expression, forskolin was used as a positive control as forskolin increase CgB and SgII mRNA synthesis in non-cardiac cells.[199] We also measured proBNP mRNA levels as several of the included ligands (norepinephrine, angiotensin II, endothelin-1, etc) are known to increase

cardiomyocyte proBNP synthesis.[42] Cells stimulated with PBS served as the control and data are presented as the change vs. levels in PBS stimulated cells (fold change).

In paper #4, we used neonatal rat cardiomyocytes to examine the effect of secretoneurin on hydrogen peroxide-induced cardiomyocyte apoptosis. For these experiments, cells were first exposed to hydrogen peroxide for 24 h, after which a proportion of the cells were co-incubated with secretoneurin and hydrogen peroxide for 24 h. Cardiomyocyte apoptosis was measured by the Cell Death Enzyme-linked Immunosorbent Assay (Cell Death Detection ELISA^{PLUS}, Roche, Basel, Switzerland) which detects DNA fragmentation by utilizing monoclonal antibodies against DNA and histones, respectively. This system has previously also been used by other groups to assess cardiomyocyte apoptosis.[200] We compared results in cells incubated with hydrogen peroxide and secretoneurin to cells that were only incubated with hydrogen peroxide.

Finally, in paper #4 neonatal cardiomyocytes were also used to examine the effect by short-term secretoneurin stimulation on phosphorylation of Akt, Erk1/2 and Stat3. In our experiments, we incubated cardiomyocytes for 10 or 30 min with secretoneurin and then quantitated the phosphorylation status of Akt, Erk1/2 and Stat3, which are the active forms of these intracellular signaling molecules.[201-203] The effect by secretoneurin was determined by comparing phosphorylation status after secretoneurin incubation to baseline p-Akt, p-Erk1/2, and p-Stat3 levels.

Ischemia-reperfusion model of the isolated perfused rat heart

The isolated perfused heart model was introduced by the German physician Oskar Langendorff in the late 19th century.[204] In the Langendorff model, the heart is excised and then perfused in a retrograde fashion via the aorta, which causes the aortic valves to shut. After closure of the aortic valves, oxygen and nutrient rich perfusate is propelled into the coronary arteries. We used a standard perfusate (Krebs-Henseleit Buffer, KHB) in our experiments.

Removing the heart from the body can terminally damage the organ and we therefore assessed the competence of all hearts before the start of the experimental protocol. Hearts with LV systolic pressure ≤100 mmHg, coronary flow ≤8 or ≥20 mL/min, heart rate ≤220 beats per minute before ischemia, or irreversible arrhythmias for more than 30 min during reperfusion were all excluded. To control for the effect of the model itself on myocardial function,[205] we implemented a 40 min stabilization period prior to the 30 min of global ischemia. After the ischemia, hearts were reperfused for 120 min before the hearts were cut in several slices, including segments that were incubated in 1% triphenyltetrazoliumchloride (TTC). The TTC stained slices were gently pressed between two glass plates and photographed. Secretoneurin was added to the perfusate in the secretoneurin group 20 min prior to induction of ischemia and throughout the reperfusion method. The end point of the study was infarct size that was calculated in the TTC slices by an independent researcher with no knowledge of treatment groups.

Real-time reverse transcription polymerase chain reaction (RT-qPCR)

Ribonucleotide acids (RNA) are single or double stranded chains of nucleotides that consist of ribose sugar, a phosphate group, and one of the four bases adenine (A), cytosine (C), guanine (G), or uracil (U). A special strain of RNA is mRNA, which is responsible for the transfer of information from the DNA double strand to protein. This process starts with the enzyme RNA polymerase binding to the DNA strand in the promoter region upstream of the target gene. RNA polymerase then proceeds in the 5'→3' direction to synthesize mRNA with DNA as the template.[206] The promoter region contains several binding sites for transcription factors which regulate mRNA synthesis, thus mRNA production is tightly controlled by extra- and intracellular signals.[199] After completion of mRNA synthesis, the mature mRNA transcript is transported out of the nucleus to the cytoplasm.[206] In the cytoplasm, by interacting with transfer RNA and ribosomes, the mRNA strand is translated into a protein as every 3 nucleotides constitute a codon representing the code for a specific amino acid.

The number of mRNA copies represents the activity of specific genes. The current method of choice to quantify mRNA levels is real-time reverse transcription polymerase chain reaction (RT-qPCR), which is a combination of three steps.[207,208] First, total RNA is

extracted from samples and the concentration and quality of RNA assessed as described in paper #3. We only used RNA with RNA integrity number (RIN)>8 for all samples. The next step is to synthesize the complementary (c)DNA strand before any measurements can be performed. After cDNA synthesis, we used a primer-and-probe based RT-qPCR system (TaqMan assays) from Applied Biosystems (Foster City, CA, USA) to quantify the mRNA levels (step 3).

In TaqMan assays, the forward and reverse primers bind to the template cDNA at the 3' end of the 3'-5' strand and the 5'-3' strand respectively, while the probe binds to a complementary sequence between the forward and reverse primers (annealing). The probe is labeled with a reporter dye at one end and a quencher due at the other end which absorbs the report dye fluorescent emission.[207] During elongation of the new DNA strand (extension), the probe is cleaved and the fluorescent dye is separated from the quencher. This increases the report dye fluorescent emission spectra which is detected by the thermo cycler.[207] A new cycle is initiated by increasing the temperature of the thermo cycler, which will separate the two DNA strands and make them available for a new cycle of annealing and extension. In RT-qPCR experiments, a doubling in DNA strands is optimally obtained per cycle. Several rounds of annealing, extension, and denaturation are needed to reach the lower limit of detection (threshold). The quantity of mRNA in the original sample will determine the number of cycles needed to reach the threshold, and genes with low number of copies will require more amplification to reach the threshold compared to more abundant genes (e.g. will have a higher C_t= threshold cycle/ C_q= crossing point).[207,208]

A criterion of great importance for RT-qPCR is the efficiency of the qPCR reaction, which should always be verified by checking the standard curve.[208] In our experiments, the efficiency was acceptable for all runs and we only used pre-designed assays which have been validated by the producer. Also to ensure stability across different extractions and RT-qPCR runs, we included RPL4 analysis in the protocol as this gene is not altered in HF (housekeeping gene).[209] We found no difference in RPL4 gene expression between HF and sham animals in our experiments.

1-D gel electrophoresis and immunoblotting

We used sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) according to the Bradford method[210] to quantitate and study processing of proteins in our experimental models. As reported in the supplementary materials of papers #3 and #4, first total protein homogenate is extracted from the samples. SDS is added to the total protein homogenate before gel loading, binds to the proteins, and gives the proteins identical charge per unit mass. After extraction, denatured sample proteins are loaded onto a gel electrophoresis system connected to a power supply. When the power is turned on, an electric field is applied across the gel and the proteins will migrate in the gel towards the positive charged anode. Small proteins will travel faster towards the anode compared to larger proteins. Of note, the granin proteins migrate anomalously on the SDS gel due to a relatively high content of acidic residues that will interact with the SDS in the gel matrix.[80] In addition, post-translational modifications will influence the number and position of bands on the gel, which we demonstrate for SgII in paper #4.

After separation of the proteins on the gel, two main strategies can be used to visualize the proteins. Staining the gel with Coomassie blue or silver staining is an unspecific method that will detect all proteins in the gel. Visible distinct bands will represent either abundant expressed proteins or represent a mix of several different proteins with similar molecular mass. To characterize the individual proteins after Coomassie blue or silver staining, the bands must be cut out from the gel and the amino acid sequence determined by mass spectrometry. However, as mass spectrometry can be time consuming and technically challenging, immunoblotting represents a more direct method to identify target proteins. In immunoblotting, the first step is to transfer the proteins from the gel to a membrane, which typically is made of nitrocellulose or PVDF. The membrane is incubated with a specific antibody that binds to the target protein (primary antibody). To avoid unspecific binding, the membrane is blocked with 5% skimmed milk prior to incubation with the primary antibody, and washed several times after each incubation step. Thereafter, the membrane is incubated with a secondary antibody conjugated with horseradish peroxidase (HRP). The secondary antibody will bind to the primary antibody. Finally, a substrate for HRP is added to the membrane and a chemiluminescent signal will be emitted and

detected by a ccd camera. The densitometry of immunostained bands was quantified by the Multi Gauge software (Fujifilm, Tokyo, Japan).

The sensitivity and specificity of the primary antibody is crucial for the quality of the immunoblot. In paper #3, we tested the specificity of the CgB antibody by co-incubating the membrane with a blocking peptide and by mapping the binding site of the antibody as previously reported.[211] No blocking peptide was available for our SgII antibody, but our results in HF animals are supported by data from SgII radioimmunoassay and the data on the proteases PC1/3 and PC2, which are increased in HF.

Enzyme-Linked ImmunoSorbent Assay

We used a commercial enzyme-linked immunosorbent assay (ELISA) for CgA measurements for papers #1 and #2 (K0025, DakoCytomation, Glostrup, Denmark).[100] In this double antibody sandwich assay, a known quantity of the capture antibody is affixed to the surface of the wells and will bind the antigen of the sample. In the next step, a detection antibody is added that binds the antigen of the capture antibody-antigen complex, followed by an enzyme-linked secondary antibody which will bind to the detection antibody. In the last step, substrate is added that the enzyme can convert to a detectable signal. Thus in the case of fluorescence ELISA, when light of the appropriate wave length is shone upon the sample, any antigen/antibody complexes will fluoresce so that the amount of antigen in the sample can be determined through the magnitude of the fluorescence. For quality control, measurements are also carried out on standard samples with known concentrations of protein to assess sensitivity and analytical stability. The analytical characteristics of an assay are normally presented by reporting the lower limit of detection and the coefficient of variation (CV). The CV is calculated by dividing the standard deviation by the mean, which should be established by measuring the same sample over time. Preferably, to demonstrate stability across the spectrum of analytical range, the CV should be reported for different concentrations of the assay. The limit of detection of the CgA assay is 7.0 U/L and the upper reference limit according to the manufacturer is 18 U/L. The intra- and interassay coefficients of variation are 5 and 10%, respectively.

Radioimmunoassay

We used radioimmunoassay (RIA) to measure granin proteins in the circulation and tissue in the papers #3 and #4. To make a RIA, the first step is to attach a gamma-radioactive isotope to a known quantity of the protein.[212-214] The radio-labeled protein is then mixed with a known amount of the antibody which will result in protein-antibody interaction. The next step is to incubate the sample with an unknown quantity of that same protein. This causes the unlabeled (or "cold") protein from the sample to compete with the radio labeled protein for the antibody binding site. As the concentration of "cold" antigen is increased, more of this protein will bind to the antibody, and thus displace the radio labeled protein. After several rounds of washing, only protein bound to antibody is retained in the well and the radioactivity of the free protein can be measured in the supernatant. A binding curve can be plotted based on the concentration of radioactivity in the supernatant and the protein levels of the original sample estimated.

To measure the granin proteins, a commercial RIA was available for CgA analysis (EuroDiagnostica AB, Malmö, Sweden),[100] while we used two in-house RIAs for CgB[213] and SgII analysis[214] (from Mats Stridsberg, MD, PhD, University of Uppsala, Uppsala, Sweden). The detection limits and the CVs of these assays are reported in paper #4.

Immunohistochemistry

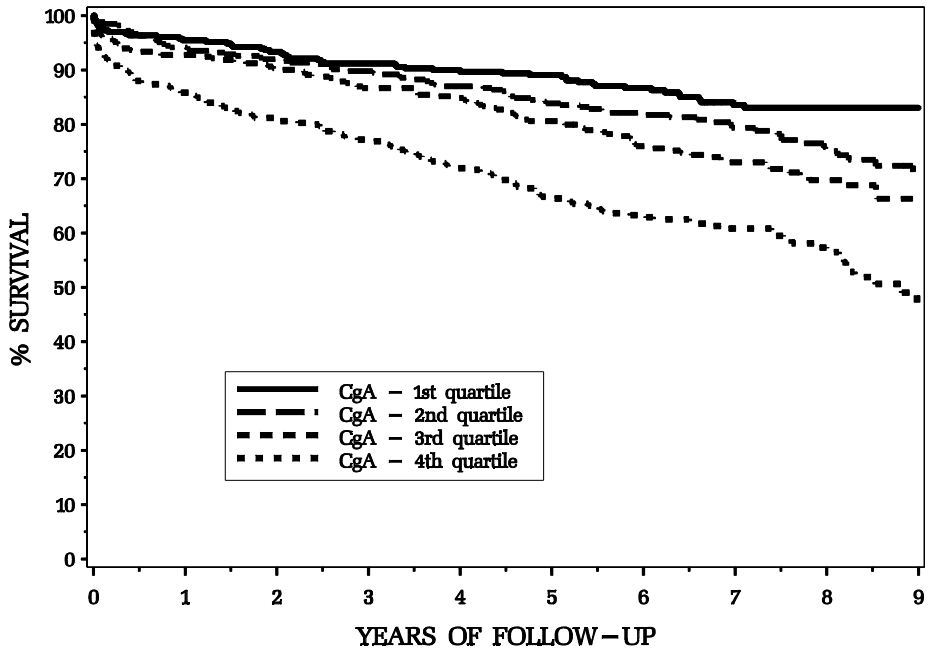
We used immunohistochemistry in paper #3 and #4 to determine the cellular localization of the granin proteins in the myocardium. Tissue samples for immunohistochemistry were first incubated overnight in 4% formalin, then washed in 30% ethanol, and later stored in 70% ethanol at 4°C before use. Slides for immunohistochemistry were prepared by standard methods. After incubation with the primary and secondary antibody, the avidin-biotin-peroxidase system (Vectastain Elite kit, Vector Laboratories, Burlingame, CA, USA) was used to further amplify immunoreactivity, before sections were counter-stained with hematoxylin. We used non-immune rabbit serum or omitted the primary antibody to check for unspecific staining.

Summery of results

Paper #I:

Circulating CgA levels were measured in 1268 patients admitted with ACS, out of whom 531 patients (42%) were diagnosed with STEMI, 447 patients (35%) diagnosed with NSTEMI, and 290 patients (23%) diagnosed with unstable angina pectoris. We found CgA levels to be correlated to several established risk factors in ACS, including age, comorbidities, proBNP levels and LVEF. During a median follow-up of 92 months (interquartile range 71-110 months), 389 (31%) of the patients died. Our principal finding in this study is an independent association between CgA levels and mortality during follow-up. This association was evident across the spectrum of ACS and also in subgroups of patients in which data on cTnT and proBNP levels and LVEF were available.

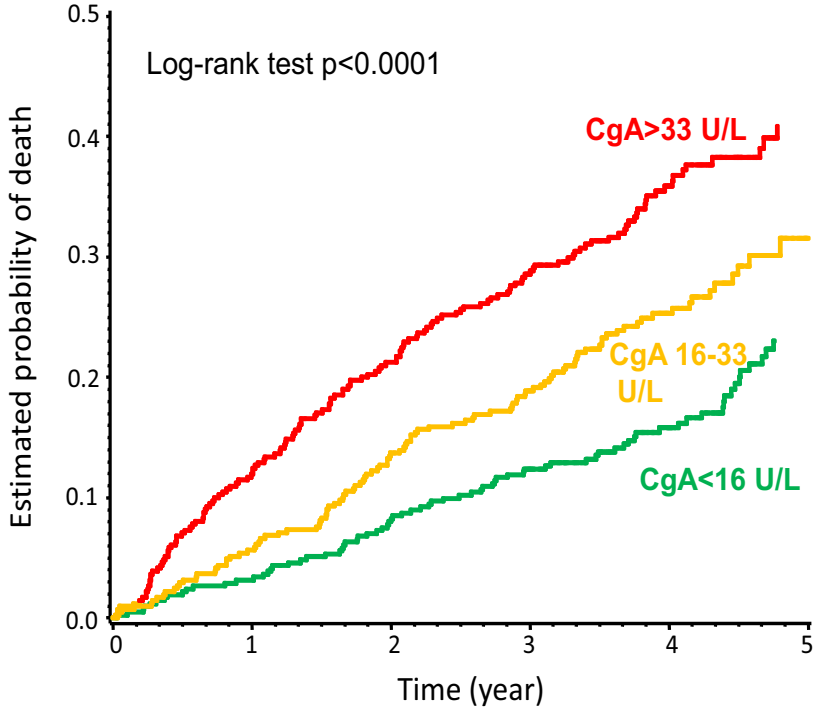
Fig 9. Association between chromogranin A levels by quartiles and long-term mortality in patients hospitalized for ACS



Paper #II:

Circulating CgA levels were measured in 1233 patients with chronic, stable HF on inclusion and after 3 months in the biomarker substudy of the GISSI-HF trial. CgA levels were associated with several established risk factors in HF, including autonomic dysfunction as measured by heart rate variability in a subgroup of patients with 24 h Holter recordings. During a median follow-up of 3.9 years (interquartile range 3.1-4.6 years), 333 (27%) of the patients died. We found that CgA levels measured on inclusion in this GISSI-HF study differentiated between patients with a poor and a favorable outcome. However, as CgA levels were influenced by several established risk factors, including age, comorbidities, blood pressure, and BNP, the association between CgA levels and mortality was attenuated and no longer significant in multivariable analysis. There were no interactions between CgA levels and rosuvastatin or n-3 polyunsaturated fatty acids therapy.

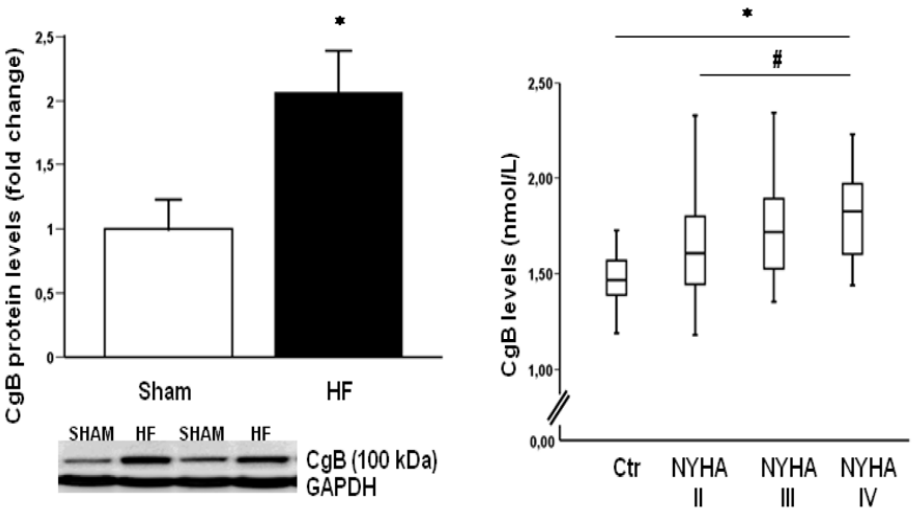
Fig 10. Association between chromogranin A levels by tertiles and long-term mortality in patients with stable HF



Paper #III:

Tissue and circulating levels of CgB in HF were examined by the use of experimental animal models and patients with HF. In a post-AMI HF mouse model, LV CgB mRNA levels were increased in proportion to HF severity as measured by lung weight. CgB protein levels were also increased in the failing myocardium, while levels were not altered in non-cardiac tissue of HF animals. We also found myocardial CgB production to be confined to the cardiomyocytes and that norepinephrine, TGF-β, and angiotensin II enhanced CgB production *in vitro*. Finally, in 80 patients with mainly stable HF, circulating CgB levels were increased compared to levels in 20 healthy age- and gender-matched control subjects. CgB levels in HF patients increased in proportion to the severity of HF as assessed by NYHA functional class.

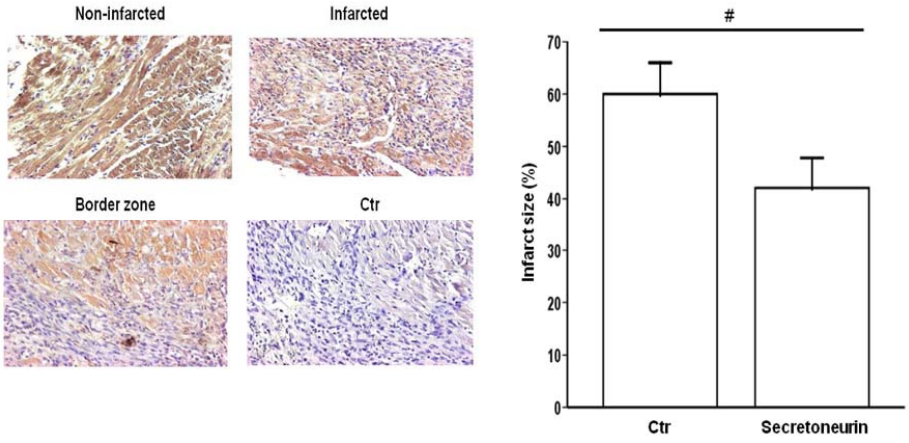
Fig 11. CgB production is increased in non-infarcted LV tissue of HF animals (left) and circulating CgB levels are increased in proportion to the severity of HF (right)



Paper #IV:

Production of SgII was examined in the post-AMI HF mouse model. SgII mRNA and protein levels were increased in the LV of HF animals, while we did not observe an increase in non-cardiac tissue during HF development. SgII production was confined to the cardiomyocytes and increased after norepinephrine and TGF-β stimulation. Circulating levels of SgII in 58 patients with chronic, stable HF of mainly mild severity were elevated compared to levels in age- and gender-matched control subjects. To explore a potential role for SgII in post-AMI HF development, a functional fragment of SgII, secretoneurin, was added to the perfusate in a global ischemia/reperfusion model of the isolated rat heart. We found that secretoneurin reduced infarct size by 30% and attenuated the increase in LV end-diastolic pressure seen after ischemia. Secretoneurin also reduced hydrogen peroxide-induced cardiomyocyte apoptosis and activated Erk1/2 and Stat3 signaling, which supports that secretoneurin plays a role as a protective peptide in HF after myocardial infarction.

Fig 12. SgII immunoreactivity (brown staining) was confined to cardiomyocytes in the myocardium (left) and the SgII fragment secretoneurin reduced infarct size after ischemia/reperfusion injury



Discussion

The main findings of this thesis are that levels of granin proteins are increased in the LV and circulation in HF and that CgA levels provide independent prognostic information in patients with ACS. The clinical usefulness of CgA as a biomarker in patients with stable HF seems more limited, although we found a close bivariate association between CgA levels and mortality.

Production of granin proteins in HF

The importance of the endocrine function of the heart in HF is well recognized.[4,38,157] Production of several proteins are increased in the failing myocardium, and we have now identified the granin protein family as a novel group of proteins that are produced by cardiomyocytes in HF. CgA has previously been shown to be localized to cardiomyocytes in HF,[109] but we now complement and transcends previous reports by demonstrating increased cardiomyocyte CgB and SgII production in HF. Recently, increased CgB production was also reported in an angiotensin II induced LV hypertrophy mice model[133] and SgII was identified in the healthy rodent myocardium,[215] which both support the validity of our results. However, by utilizing a translational approach, we are the first to demonstrate increased CgB and SgII levels in the myocardium and circulation in HF.

Characterizing the regulatory mechanism of cardiomyocyte gene expression can be assessed in experimental models.[175,193] We used isolated cardiomyocytes to examine the mechanisms responsible for increased granin production in HF. In these experiments, norepinephrine and TGF-β increased CgB and SgII mRNA levels, while angiotensin II only stimulated CgB production. In contrast, we were not able to identify factors that increased CgA mRNA levels. The difference in responsiveness of the granin genes to our panel of hormones, growth factors, and cytokines can be explained by variations in the promoter region of the granins.[199] For CgA and CgB, a cAMP responsive element has been identified as the crucial regulator of gene expression,[152,216] while a serum response element is important for SgII expression.[217] Other regions in the CgA promoter may also influence gene expression,[218] which may explain the lack of responsiveness in CgA synthesis in our system. Additional strategies should be explored to investigate CgA

production in HF that seems to be controlled by other mechanism than CgB and SgII expression.

Our data indicate that β-adrenergic signalling and the TGF-β pathway might induce CgB and SgII expression in cardiomyocytes during HF development. However, these data should be validated and further explored before any definite mechanism for CgB and SgII synthesis in the myocardium can be established. Pertinent to this, hypoxia-inducible factor-1α (HIF-1α) may also play a role after myocardial ischemia as HIF-1α increase SgII production in skeletal muscle after hypoxia.[219] The effect of hypoxia on cardiomyocyte gene expression was not examined in our models and should be explored in future studies.

HF is a syndrome that affects multiple organs of the body, not just the heart. Pulmonary tissue, liver, and the gastrointestinal tract are affected by congestion, while the inflammatory response in HF will induce changes in the spleen. Hemodynamic changes may also compromise renal function. Accordingly, to provide a comprehensive assessment of granin production in HF, we explored granin production also outside the heart in our post-AMI HF mouse model. We found the granin proteins to be expressed in all organs examined, which is new information compared to data from previous reports,[220] but there was no increase in granin expression in the right ventricle, pulmonary tissue, liver, spleen, stomach, colon, or skeletal muscle in animals with HF.

In our post-infarction HF model, we identified CgA, CgB, and SgII mRNA and protein levels as increased in the failing myocardium. In paper #4, we were also able to demonstrate enhanced processing of full-length SgII to shorter fragments. This could be important as the short post-translational modified fragments represent the functional units of SgII.[221,222] The mechanism for the increased processing in the failing myocardium seems to be elevated PC1/3 and PC2 levels, which previously have been identified as the major proteases for the granin proteins.[83,84] Of note, enhanced processing of SgII to shorter fragments in neuroendocrine cells is associated with increased secretion of SgII fragments from the cell,[223] but we have not measured SgII secretion from cardiomyocytes in HF.

We lack data on production of granin proteins in the adrenal medulla in HF, which is a limitation to our work. However, although the adrenal medulla is considered the principal organ for CgA synthesis, the concentration of SgII is much lower in the adrenal medulla,[224] and the gastrointestinal tract has been proposed as the principal organ contributing to circulating SgII levels.[221] In our HF animals, no increase in SgII levels was observed either in the stomach or in the colon. Moreover, in STEMI patients there are only weak or no correlations between CgA and catecholamine levels in the subacute phase,[162,163] hence supporting a model of several organs contributing to CgA synthesis in cardiovascular disease.[164] The same model could be relevant for CgB and SgII as these proteins are expressed throughout the body and seems to increase in the LV in cardiovascular disease. Future studies should establish the principal organs that synthesize granin proteins in cardiovascular disease by performing selective sampling across different vascular beds in patients with ACS and HF.

Of more general scientific interest, our results identify the granin proteins as proteins that are ubiquitously expressed in the body, rather than confined to the cells of the neuroendocrine system. We believe the widespread distribution, and the increase in production in cells with an endocrine phenotype, support our revised model of granin proteins as markers of cells with a high secretory rate. This could also be relevant for other conditions with a secretory phenotype during disease progression and provide a common denominator for diseases in which granin proteins are markers of disease progress and severity.

Functional aspects of granin proteins in cardiovascular disease

Proteins that are increased in patients with cardiovascular disease could influence cardiovascular pathophysiology by a direct effect on the myocardium, or through effects on extra-cardiac organs by a paracrine or endocrine mechanism.[4,12,24] The granin proteins are increased in patients with cardiovascular disease and there is a possibility that these proteins could play a causal role in disease progression.

Seminal work by other groups have documented effects by the short CgA fragments vasostatin II and catestatin on the vasculature and the myocardium.[134] These effects include enhanced vasodilatation,[137] attenuated contractility,[109,138,142] improved endothelial function,[140] reduced catecholamine release,[143] and protection from myocardial ischemia-reperfusion injury[147] and excessive β-adrenergic and endothelin-1 signaling in cardiomyocytes.[144,225] The protection of CgA against detrimental β-adrenergic drive has been proposed as the principal functional aspect of CgA in cardiovascular disease.[145,146] Interestingly, myocardial CgA expression was recently found to decrease during reverse remodeling in HF patients that had LV assist device (LVAD) implantation, while there was no change in circulating CgA levels.[226] This report supports a dynamic regulation of CgA in cardiomyocytes during cardiovascular disease, but also highlight the widespread production of CgA and the lack of specificity of circulating CgA levels for cardiovascular disease. This has also been demonstrated by other groups which found CgA to be a poor diagnostic marker for HF in patients hospitalized for dyspnea,[227] but a strong prognostic marker in the same patients.[228,229] There was no significant difference in circulating CgA levels between HF patients and healthy control subjects in paper #3 and #4 of this thesis.

Prior to our work, no information was available on the role SgII and the SgII fragment secretoneurin play in cardiovascular disease. We now provide evidence that SgII expression and processing are increased in HF and that secretoneurin may play a role in cardiovascular pathophysiology. The 30% reduction in infarct size after ischemia-reperfusion injury and the attenuated cardiomyocyte apoptosis suggest a counteractive role for secretoneurin in post-infarction HF, but long-term effects of secretoneurin should be established before the net effect of increased SgII levels in HF can be determined. This also relates to CgB, although CgB previously has been identified as important for cardiomyocyte Ca^{2+} handling and hypertrophy,[133] which are relevant in HF.[4,12,16,24] Mice with CgB deletion[123] and overexpression[230] have been reported, but no information is available on the cardiovascular phenotype of these genetically modified mice. Characterizing the cardiovascular phenotype of the CgB gain- and loss-of-function mice in the steady-state situation and during stress could provide important information on the role of CgB in the myocardium, although cardiospecific mutations would be superior to the current whole-body models that have alterations in neuroendocrine function.[123]

Granin proteins as cardiovascular biomarkers

A large number of proteins have been proposed as new cardiovascular biomarkers (Figure 4),[38,75] but currently the cardiac troponins and the BNPs are the only biomarkers that are clinically used in patients with ACS and HF.[7,9,18,75] We now propose the granin proteins as possible new cardiovascular biomarkers, but only CgA has so far been tested in large clinical cohorts.[161-163,171,172,226-229] Still, the potential of the granin proteins as cardiovascular biomarkers has recently been recognized in authoritative reviews on cardiovascular biomarkers.[38,75]

The granin proteins are reported to have a high signal-to-noise ratio and no need for strict pre-analytical handling.[99,160] These analytical aspects are imperative for a protein to have potential as a clinical useful biomarker (Figure 6).[44] Moreover, CgA seems to provide incremental prognostic information to established risk indices in patients with acute cardiovascular disease,[162,163] as also demonstrated in paper #1 of this thesis. CgA was recently also found to provide independent prognostic information in patients with acute decompensated HF[228] and in unselected patients hospitalized for dyspnea,[229] but there is still a lack of information on the ability of CgA to improve prognostic accuracy and reclassification. Future studies should also perform receiver operating statistical analysis and model fit before CgA can be considered a strong contender in the on-going cardiovascular biomarker race.[56] There is also a need to better understand the pathophysiology of CgA before this marker can be explored for guiding patient management.[70] This will require both experimental and clinical studies. The current model links CgA to β-adrenergic drive[95-97,145,146] and it should be assessed whether CgA could be useful for titration of β-adrenergic blocker therapy in patients with cardiovascular disease. However, this should first be addressed in experimental models and by propensity score statistics[231] in established clinical cohorts.

Our data identify CgA as a potential clinical relevant prognostic biomarker in the acute setting. The mechanism for the superior prognostic information of CgA in acute patients vs. stable patients could relate to a larger contribution by the adrenal medulla, and possibly the myocardium, to circulating CgA levels in the acute setting compared to stable patients. By providing integrated information on neuroendocrine activity and the

myocardium, CgA could provide information on pathophysiology currently not implemented in established risk indices. However, the association with neuroendocrine tone will reduce the potential of CgA as a diagnostic biomarker in cardiovascular disease and this has also been demonstrated by other groups.[227] Interestingly, although not a good marker for diagnosing HF in patients with dyspnea, CgA was still a powerful prognostic biomarker in the same cohort of patients.[229] CgA thus seem useful to identify a subgroup of patients with similar phenotype and for risk assessment in the acute setting, but will not help in diagnosing HF *per se*. Other groups have also found CgA[232] to provide independent prognostic information in patients with critical illness, and CgA is more a biomarker of disease severity than a marker associated with a specific organ dysfunction. We recently explored this further by demonstrating superior information by CgA over NT-proBNP and troponin T as measured by a highly sensitive (hs) assay for the prediction of hospital mortality in patients with severe sepsis.[233] Of note, in this study we also found CgA levels associated with previous cardiac disease and indices of cardiovascular stability such as cardiovascular SOFA score on day 3 and septic shock during the hospitalization. The incremental prognostic information by CgA in this study, including in analysis that adjusted for established risk scores in sepsis, indicates that CgA represents pathophysiology currently not covered by established risk indices and cardiac biomarkers. The strong and indepedent information by CgA in patients with AMI and acute HF was also supported by a post hoc analysis from the OPTIMAAL trial in which 37 circulating biomarkers have been measured.[234] By exploratory factorial analysis CgA was clustered together with 8 other biomarkers as the most powerful predictors of all-cause mortality and the combined end point of cardiovascular death or non-fatal recurrent AMI. Interestingly, there was only moderate correlations between CgA levels and the levels of the other biomarkers ($r<0.4$), thus CgA does not seem to duplicate information that can be attained from clinical examination, echocardiography, or by measuring other cardiac biomarkers. Future studies should progress our understanding of the association between CgA levels, adrenergic tone, and cardiovascular disease. Moreover, although not correlated with the increment in hs-cTnT levels during long-distance running,[235] this should not be used to prelude an association between CgA levels and cardiac burden during exercise as hs-cTnT elevations do not seem closely correlated with cardiac pathology.[236] Finally, to promote CgA as a clinical relevant biomarker, we need more

information on the association between CgA levels and medication and the merit of CgA to guide patient management.

There is minimal information currently available on CgB and SgII as biomarkers and no conclusions can be made regarding their potential in cardiovascular disease although CgB is considered an attractive candidate also by other groups.[237] We demonstrate a significant increase in circulating CgB and SgII levels in patients with HF compared to age- and gender-matched control subjects. CgB was also found to increase in proportion to the severity of HF as assessed by the NYHA functional class. There was a more robust increase of CgB and SgII levels over CgA levels in the HF patients of papers #3 and #4, but whether this relates to a larger contribution by the myocardium to circulating CgB and SgII levels remains to be established. Future studies should focus on the pathophysiology of these proteins in cardiovascular disease and the diagnostic and prognostic merit in ACS and HF patients. Although we find that the SgII fragment secretoneurin attenuates ischemia-reperfusion injury and cardiomyocyte apoptosis, this does not preclude that additional pathophysiology could be more relevant for SgII as a cardiovascular biomarker. Pertinent to this, our data in isolated cardiomyocytes link SgII expression to norepinephrine and TGF-β signalling, while angiotensin-II stimulation also increased CgB mRNA levels.

The role CgB plays in cardiovascular disease is not established. Previous work have linked CgB to Ca^{2+} handling and LV hypertrophy in the myocardium,[133] but the mechanism for, and the net effect of, elevated CgB levels in HF have yet to be determined. The widespread production of CgB and SgII in the body (papers #3 and #4) also suggests that these proteins could be markers of extra-cardiac pathophysiology, including adrenergic tone that has been demonstrated for CgA. In contrast, there does not seem to be a close correlation between BNP and granin levels in patients with HF (paper #4). More surprisingly, the individual granin proteins neither seem to be closely correlated in patients with HF (paper #4). Accordingly, the granin proteins should be considered separate biomarkers in cardiovascular disease, and they do not appear to duplicate the information from BNP in HF patients. Future research, which should combine clinical studies and experimental work in cells and animals, will increase our understanding of the

pathophysiology of these proteins and provide more data on CgA, CgB, and SgII as new cardiovascular biomarkers.

Conclusion

We have demonstrated that CgA provides independent prognostic information in patients with ACS (paper #1), while the role of CgA as a biomarker in patients with stable HF seems more limited (paper #2). The two other principal granin proteins, CgB and SgII, are increased in the LV and circulation in HF (paper #3 and #4), but currently limited information is available concerning the functional aspects and the utility of these proteins as cardiovascular biomarkers. The SgII derived fragment secretoneurin protects against myocardial ischemia-reperfusion injury and cardiomyocyte apoptosis, but long-term effects of elevated SgII levels in HF have not been assessed. Accordingly, although our data identify CgA, CgB, and SgII as an interesting group of proteins in cardiovascular disease, more basic and clinical research are needed to determine the pathophysiological role these proteins play in ACS and HF and the potential of the granin proteins as cardiovascular biomarkers.

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Prognostic value of circulating chromogranin A levels in acute coronary syndromes

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Aims To determine whether circulating levels of chromogranin A (CgA) provide prognostic information independently of conventional risk markers in acute coronary syndromes (ACSs).

Methods and results We measured circulating CgA levels on day 1 in 1268 patients (median age 67 years, 70% male) with ACS admitted to a single coronary care unit of a Scandinavian teaching hospital. The merit of CgA as a biomarker was evaluated after adjusting for conventional cardiovascular risk factors. During a median follow-up of 92 months, 389 patients (31%) died. The baseline CgA concentration was strongly associated with increased long-term mortality [hazard ratio per 1 standard deviation increase in logarithmically transformed CgA level: 1.57 (1.44–1.70), $P < 0.001$], heart failure hospitalizations [1.54 (1.35–1.76), $P < 0.001$], and recurrent myocardial infarction (MI) [1.27 (1.10–1.47), $P < 0.001$], but not stroke. After adjustment for conventional cardiovascular risk markers, the association remained significant for mortality [hazard ratio 1.28 (1.15–1.42), $P < 0.001$] and heart failure hospitalization [hazard ratio 1.24 (1.04–1.47), $P = 0.02$], but not recurrent MI.

Conclusion CgA is an independent predictor of long-term mortality and heart failure hospitalizations across the spectrum of ACSs and provides incremental prognostic information to conventional cardiovascular risk markers.

Keywords Acute coronary syndromes • Chromogranin A • Troponin T • Echocardiography • Prognosis

During the past decade, major progress has been made in the management of patients with acute coronary syndromes (ACSs). In parallel with advances in medical therapy and increasing use of an early invasive strategy, there has been focus on early risk stratification of patients, and in particular, the potential prognostic utility of circulating biomarkers.[1] Currently, cardiac-specific troponins and B-type natriuretic peptide are the major routinely measured circulating biomarkers in patients with ACSs.[2,3]

Chromogranin A (CgA) is a 439 amino acid, 49 kDa polypeptide, which has been identified throughout the endocrine and nervous systems.[4] Markedly elevated plasma levels have been observed in patients with neuroendocrine tumours,[5] such as pheochromocytoma[6] and carcinoid,[7] and the clinical application of CgA measurements has so far been limited to diagnosis and follow-up of patients with such tumours. However, circulating CgA levels also correlate closely with increased sympathetic activity both in the adrenal medulla and the peripheral nerve endings,[8–10] suggesting that circulating CgA may integrate neuroendocrine signals from various sources and thus represent an index of overall neuroendocrine activity. Moreover, myocardial production of CgA in humans with dilated and hypertrophic cardiomyopathy has recently been demonstrated,[11] and CgA has been shown to increase in

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proportion to clinical severity and to be associated with prognosis in patients with both chronic and post-infarction heart failure.[12,13] In a small population with predominantly ST-elevation myocardial infarction (MI), we have previously reported a univariable association between CgA levels and long-term survival,[14] but because of modest study power, it remains unclear whether CgA is an independent predictor of survival. In patients with unstable angina and non-ST-elevation MI, no prognostic data are currently available. Because increased neuroendocrine activity may be related to potentially harmful pathophysiological processes in patients with both ST-elevation and non-ST-elevation ACS, including endothelial dysfunction and activation of pro-inflammatory cytokines, we hypothesized that circulating CgA levels would be predictive of the incidence of death and non-fatal cardiovascular events across the spectrum of ACS and would provide prognostic information independently of conventional risk markers, including objective measures of left ventricular dysfunction and contemporary cardiac biomarkers.

Methods

Study design

Patients with ACS, defined as a diagnosis of unstable angina, non-ST-elevation MI, or ST-elevation MI, admitted to the coronary care unit (CCU) of the Sahlgrenska University Hospital, Gothenburg, Sweden during the period mid-September 1995 to mid-March 2001, were eligible for participation in a prospective risk stratification programme, PRACSIS (Prognosis and Risk in Acute Coronary Syndromes in Sweden),[15] in which the main study exclusion criteria were age <18 or ≥ 80 years, non-coronary artery disease associated with a life expectancy <1 year, residence outside the city of Gothenburg, unwillingness to participate, and prior admission resulting in inclusion in the study.

During this 5.5-year period, a total of 2335 patients were included in the PRACSIS programme. Until November 1995, only clinical information was collected in PRACSIS and we did not perform consecutive serum sampling, resulting in only six random morning pilot serum samples being drawn in this period. Thereafter, serum for later analysis was obtained the first morning after admission to the CCU in the patients who, at this stage, were assigned a diagnosis of ACS. Thus, a number of patients were not eligible for blood sampling despite later being considered as having ACS and noted as such. Another portion of patients ($n = 612$) in PRACSIS were transferred to the CCU from an internal medicine ward where they had been admitted owing to an initially uncertain ACS, or from the intensive care unit where they were admitted owing to the need of mechanical ventilation. We lack serum from a majority of these patients. Yet another portion of patients did not survive until the first morning in hospital or were at this time undergoing angiography, and during some holidays serum sampling was not attempted. These patients were included in the PRACSIS programme, but not in the biomarker substudy. Users of proton pump inhibitors on admission ($n = 38$) were also excluded from this study, as proton pump inhibitors are known to increase circulating CgA levels.[16] Thus, the final study group comprised 1268 patients.

The primary outcome measure was mortality from all causes. The median follow-up for this primary endpoint was 92 (interquartile range 71–110) months (until 1 January 2007). Survival confirmation and date of death were obtained from the Swedish National Population Registry. Eleven patients, who emigrated from Sweden, were lost to follow-up and censored at the day of emigration.

Pre-specified secondary outcome measures were the incidence of the following separate morbidity endpoints: heart failure [International Statistical Classification of Disease, Ninth Revision (ICD-9) code 428 or ICD-10 code I50], acute MI (ICD-9 code 410 or ICD-10 code I21 or I22), and stroke (ICD-9 codes 431, 432, 433, or 436 or ICD-10 codes I61, I62, I63, or I64). These data were obtained from the Swedish Hospital Discharge Register. Because of a slower confirmation process than for mortality data, morbidity data were not available after 31 December 2002. Accordingly, the median follow-up period for morbidity data was 50 (interquartile range 32–65) months. For quality control purposes, morbidity data from the Registry were checked against information in the patients' medical records by a cardiologist (M.H.) blinded to biomarker results. No patient was excluded owing to missing data for outcome.

Patients were prospectively classified according to Killip class on admission and during the index hospitalization. Electrocardiographic findings on admission were classified according to the presence or absence of ST-segment elevation and ST-segment depression. On the basis of hospital records and personal interview, patients were classified as having or not having a history of MI, angina pectoris, chronic heart failure, diabetes mellitus, or hypertension. The study protocol was approved by the Regional Ethics Committee before the initiation of the study. Informed consent was obtained from all participating patients.

Blood sampling procedures and echocardiography

Peripheral venous blood was obtained within 24 h of admission by direct venipuncture of an antecubital vein after the patients had been supine for >30 min. Blood samples for CgA determination were drawn into serum tubes and centrifuged within 1 h. Blood samples for the determination of pro-B-type natriuretic peptide (proBNP) were drawn into pyrogen-free tubes with EDTA as anticoagulant, immediately immersed in ice water, and centrifuged within 1 h.

All serum samples were stored at -70°C pending analysis. Plasma and serum samples had been thawed twice prior to CgA analysis. However, CgA is considered to be stable *in vitro* at room temperature and plasma levels are not influenced by repeated thawing–refreezing cycles.[17] Echocardiographic investigation was performed by an experienced investigator within 5 days of hospital admission, as described previously.[18,19]

Biochemical analyses

CgA in serum was measured by a commercially available ELISA assay (code K0025, DakoCytomation, Glostrup, Denmark). The detection limit of the assay was 7.0 U/L, and the intra- and interassay coefficients of variance were <5 and 10%, respectively. According to the manufacturer, the upper reference limit is 18 U/L. Troponin T and creatine kinase MB fraction in serum were measured on a modular platform (Roche Diagnostics, Mannheim, Germany). Troponin T levels were unavailable in 225 subjects, as troponin T measurement was not part of the clinical routine during the first inclusion period. $ProBNP_{3-108}$ was measured using immunofluorescent assays calibrated with spiked plasma (Biosite Inc., San Diego, CA, USA).[20] The minimal detectable concentration was 400 ng/L and the upper range 30 000 ng/L. All samples were run in duplicate in a blinded fashion. Creatinine and total cholesterol concentrations in serum were determined by routine laboratory methods. Creatinine clearance rate (mL/min) was estimated (estimated glomerular filtration rate, eGFR) using the Cockcroft–Gault formula,[21] as [(140 − age) × weight (kg)/serum creatinine (μmol/L)] multiplied by a constant of 1.23 in men and 1.04 in women.

Statistical methods

Categorical variables were reported as proportions and continuous variables as median or mean values. The association between CgA and baseline demographic variables and cardiovascular risk factors was assessed by the Mann–Whitney *U* test and Spearman rank correlation (r_s) for categorical and continuous variables, respectively. To visualize the relationship between CgA quartiles and mortality, Kaplan–Meier plots were generated. Cox proportional hazards regression analyses were used to calculate crude and adjusted risk estimates associated with a 1 standard deviation (SD) increase in logarithmically transformed CgA levels for the primary endpoint: mortality from all causes, as well as for the following individual secondary endpoints: hospitalizations for heart failure, recurrent MI, and stroke. Adjustments were made for the following confounders: age (continuous), gender, index diagnosis, smoking status, prior MI, angina pectoris, diabetes, hypertension, heart failure, Killip class (dichotomous, i.e. cutoff Killip class >1), eGFR (continuous, logarithmically transformed), heart rate (continuous, logarithmically transformed), and peak creatine kinase-MB (continuous, logarithmically transformed). In addition, adjustments were also made for troponin T, left ventricular ejection fraction, and proBNP (all continuous and logarithmically transformed) in the cohorts where such measurements were available.

The assumption of proportional hazards was assessed by studying whether interaction terms between the logarithm of time and covariates significantly improved the −2 log-likelihood of the model. The assumption was met for all variables in all models, except for the endpoint rehospitalization owing to heart failure, where previous MI and creatine kinase-MB showed a slight non-proportionality in the total cohort, and index diagnosis and creatine kinase-MB in the cohort with troponin T measurements available. Inclusion of the time-dependent covariates into the corresponding models above resulted in only minor changes of the hazard ratios for CgA. We therefore decided to use the original models in order to cohere with our published reports on other markers from the same cohorts and to adjust for the same covariates in the different endpoint analyses.

Similarly, the assumption of linearity for continuous variables was checked by entering the squared transformations of the variables into the models. A significant change in the −2 log-likelihood for any model was considered a sign of non-linearity. All variables met the assumption of linearity in all models, except for age, regarding the endpoint rehospitalization owing to MI in the total cohort and regarding rehospitalization owing to stroke in the three other cohorts. Also, eGFR showed sign of non-linearity regarding heart failure in the cohort where troponin T, ejection fraction, and proBNP were available. For these models, we analysed the hazard ratios for CgA when the corresponding transformations were entered into the model, which resulted in only small changes from the original models, and, for the same reasons as for non-proportionality, we decided to use the models without squared transformation of these covariates.

Our primary objective variable CgA did not show any sign of non-proportionality or non-linearity.

Hazard ratios are given with 95% confidence intervals. All *P*-values are two-tailed and considered significant if <0.05.

Results

Baseline characteristics

A total of 1268 patients (median age 67 years, 70% male) had blood samples for CgA determination obtained within 24 h of admission and were not users of proton pump inhibitors at the time of admission. The baseline characteristics of patients according to CgA quartiles are presented in *Table 1*, where also data on the entire PRACSIS population are given for comparison. Patients with higher CgA values were more likely to be older, to have lower body mass index, to have clinical evidence of heart failure, a history of MI, angina, congestive heart failure, or diabetes mellitus; to be diuretic users, angiotensin-converting enzyme-inhibitor or angiotensin receptor blocker users, statin users, or aspirin users (data not shown); and to have a low ejection fraction or low eGFR. There was no relation between CgA and troponin T or creatine kinase MB fraction in serum. A significant correlation ($r_s = -0.43$, $P < 0.001$) between eGFR and CgA indicated that renal function influenced the CgA level. On the other hand, the lack of correlation between CgA and troponin T values ($r_s = 0.03$, $P = 0.18$) indicated that myocardial necrosis was not a major explanation for increased CgA levels. There were no significant differences in CgA levels between female and male patients. There was no significant interaction between index diagnosis and CgA regarding outcome. Accordingly, we decided not to analyse these groups separately.

Chromogranin A and long-term mortality

During a median follow-up of 92 months (interquartile range 71–110 months), 389 patients died. CgA serum levels at baseline were closely associated with long-term, all-cause mortality [hazard ratio per 1 SD increase in logarithmically transformed CgA levels: 1.57 (1.44–1.70), $P < 0.001$]. The Kaplan–Meier survival curves by CgA quartiles are depicted in *Figure 1*. After adjustment for conventional risk factors, CgA remained independently associated with mortality [hazard ratio per 1 SD increase in logarithmically transformed CgA levels: 1.28 (1.15–1.42), $P < 0.001$] (*Table 2*). CgA levels were also an independent predictor of mortality in the subgroup of patients in whom troponin T levels were available and adjusted for in addition to the covariates in the first model [$n = 1043$; HR 1.27 (1.13–1.42), $P < 0.001$]. In the group where left ventricular ejection fraction was determined ($n = 824$), CgA was as an independent predictor of all-cause mortality after adjustment for conventional cardiovascular risk factors, troponin T levels, and echocardiographically assessed left ventricular ejection fraction [HR 1.26 (1.10–1.44), $P < 0.001$]. In the group where also data on proBNP were available and additionally adjusted for ($n = 709$), a significant predictive value of CgA was also noted [HR 1.18 (1.01–1.37), $P = 0.04$]. Patients with both CgA and proBNP in the highest quartiles had an especially poor prognosis (*Figure 2*).

Chromogranin A and non-fatal cardiovascular events

By univariable analyses, the baseline CgA concentration was strongly associated with the incidence of heart failure hospitalizations [hazard ratio 1.54 (1.35–1.76), $P < 0.001$] and recurrent MI [hazard ratio 1.27 (1.10–1.47), $P < 0.001$], but not stroke [hazard ratio 1.16 (0.93–1.46), $P = 0.19$] (*Table 2*). After adjustment for conventional risk factors, CgA remained independently associated with the incidence of heart failure hospitalizations [hazard ratio 1.24

Table 1 Patient characteristics according to chromogranin A (U/L) quartile

	CgA ≤14.7 ($n = 320$)	CgA 14.8–20.9 ($n = 315$)	CgA 21.0–33.7 ($n = 318$)	CgA >33.7 ($n = 315$)	*P*-value[a]	Entire population[b] ($n = 2258$)
Age (years)	60 ± 11	65 ± 10	67 ± 9	68 ± 9	<0.001	66 ± 10
Female	89 (28)	98 (31)	90 (28)	102 (32)	0.31	688 (30)
Previous MI	57 (18)	65 (21)	68 (21)	84 (27)	0.002	558 (25)
Previous angina	125 (39)	149 (47)	143 (45)	152 (48)	0.02	1173 (52)
Previous heart failure	14 (4)	26 (8)	19 (6)	43 (14)	<0.001	233 (10)
Previous diabetes	50 (16)	46 (15)	42 (13)	75 (24)	0.01	435 (19)
Previous hypertension (1)[c]	134 (42)	118 (38)	109 (34)	138 (44)	0.83	938 (42)
Previous hypercholesterolaemia (1)[c]	91 (29)	96 (30)	81 (25)	87 (28)	0.41	665 (30)
Current smoker (20)[c]	112 (35)	95 (31)	96 (30)	97 (32)	0.24	648 (30)
ST-elevation MI	137 (43)	121 (38)	134 (42)	139 (44)	0.64	840 (37)
Non-ST-elevation MI	108 (34)	111 (35)	116 (36)	112 (36)	0.54	766 (34)
Unstable angina	75 (23)	83 (26)	68 (21)	64 (20)	0.21	652 (29)
ST-elevation on admission (2)[c]	128 (40)	109 (35)	119 (37)	121 (39)	0.77	744 (33)
ST-depression (no elevation) on admission (2)[c]	32 (10)	35 (11)	33 (10)	43 (14)	0.11	253 (11)
Q-wave on admission (2)[c]	39 (12)	19 (6)	38 (12)	53 (17)	0.01	236 (10)
SBP <100 mmHg on admission (1)[c]	6 (2)	13 (4)	9 (3)	13 (4)	0.23	95 (4)
Heart rate on admission (b.p.m.) (2)[c]	76 ± 19	76 ± 22	75 ± 20	77 ± 21	0.94	77 ± 22
CK-MB max (μg/L)	56 (8, 211)	49 (7, 148)	60 (11, 78)	62 (10, 203)	0.36	38 (5, 150)
Troponin T max (μg/L) (225)[c,d]	0.8 (0.1, 3.9)	0.8 (0.0, 3.4)	1.2 (0.1, 4.3)	0.8 (0.1, 4.1)	0.18	0.6 (0.0, 3.4)
eGFR (mL/min) (19)[c]	82 ± 23	70 ± 20	65 ± 20	56 ± 21	<0.001	67 ± 24
proBNP (ng/L) (265)[c]	1327 (400, 2517)	1551 (572, 3007)	1982 (929, 3572)	2258 (1018, 4307)	<0.001	1772 (702, 3238)
Body mass index (kg/m^2) (32)[c]	27.2 ± 4.0	26.3 ± 3.8	25.8 ± 3.7	25.5 ± 4.0	<0.001	26.3 ± 3.9
Killip class II–IV on admission (2)[c]	11 (3)	18 (6)	18 (6)	37 (12)	<0.001	193 (9)
Max Killip class II–IV(2)[c]	33 (10)	47 (15)	65 (20)	85 (27)	<0.001	463 (21)
Thrombolysis/primary PCI	109 (34)	99 (31)	100 (31)	107 (34)	0.98	600 (27)
Other PCI or CABG during hospitalization	88 (28)	92 (29)	90 (28)	71 (23)	0.12	669 (30)
LV ejection fraction (%) (278)[c]	54 ± 11	54 ± 12	51 ± 12	50 ± 13	<0.001	52 ± 13

Data expressed as *n* (%), mean ± SD, or median (25th, 75th percentile).
CABG, coronary artery bypass grafting; CK-MB, creatine kinase MB fraction; LV, left ventricular; PCI, percutaneous coronary intervention; SBP, systolic blood pressure.
[a]Actual CgA value used in *P*-value calculations.
[b]All ACS patients admitted without proton pump inhibitors during inclusion period.
[c]Number of CgA patients where information was missing.
[d]The troponin T level was below detection in 22% of patients ($n = 64, 64, 53, 53$ in the CgA quartiles given above).

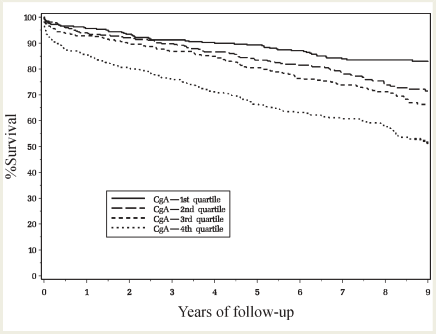

Figure 1 Association between chromogranin A (CgA) levels by quartiles and all-cause mortality in patients with acute coronary syndromes.

(1.04–1.47), $P = 0.02$], whereas the association with recurrent MI was attenuated [hazard ratio 1.15 (0.96–1.36), $P = 0.12$] (*Table 2*). In the subgroup where troponin T was available and adjusted for, CgA was significantly associated with both the incidence of heart failure ($P = 0.04$) and MI ($P = 0.04$). However, in the subsample of patients with echocardiographic data ($n = 824$), these associations were attenuated and no longer significant after adjustment for left ventricular ejection fraction (*Table 2*).

Discussion

The new information obtained from the present study is that plasma levels of CgA in the acute phase proved to be an independent predictor of all-cause mortality in patients with ACSs after adjustment for conventional risk factors, troponin T levels, echocardiographically assessed left ventricular ejection fraction, and proBNP. CgA levels were also associated with heart failure hospitalizations during follow-up independently of conventional risk factors, including troponin T. However, in the subsample of

Table 2 Associations between chromogranin A concentrations and events during follow-up in patients with acute coronary syndrome

Endpoint	Unadjusted		Adjusted	
	HR[a] (95% CI)	*P*-value	HR[a] (95% CI)	*P*-value
Total cohort ($n = 1268$)				
Mortality	1.57 (1.44, 1.70)	<0.001	1.28 (1.15, 1.42)[b]	<0.001[b]
Heart failure	1.54 (1.35, 1.76)	<0.001	1.24 (1.04, 1.47)[b]	0.02[b]
Recurrent MI	1.27 (1.10, 1.47)	<0.001	1.15 (0.96, 1.36)[b]	0.12[b]
Stroke	1.16 (0.93, 1.46)	0.19	0.96 (0.73, 1.26)[b]	0.76[b]
With troponin T ($n = 1043$)				
Mortality	1.56 (1.43, 1.71)	<0.001	1.27 (1.13, 1.42)[c]	<0.001[c]
Heart failure	1.46 (1.26, 1.71)	<0.001	1.23 (1.01, 1.49)[c]	0.04[c]
Recurrent MI	1.31 (1.12, 1.52)	<0.001	1.21 (1.00, 1.47)[c]	0.04[c]
Stroke	1.18 (0.92, 1.51)	0.19	0.96 (0.71, 1.29)[c]	0.77[c]
With troponin T and LV ejection fraction ($n = 824$)				
Mortality	1.56 (1.41, 1.74)	<0.001	1.26 (1.10, 1.44)[d]	<0.001[d]
Heart failure	1.33 (1.10, 1.61)	0.004	1.12 (0.88, 1.42)[d]	0.36[d]
Recurrent MI	1.31 (1.10, 1.57)	0.003	1.17 (0.95, 1.45)[d]	0.14[d]
Stroke	1.16 (0.86, 1.57)	0.34	0.91 (0.62, 1.33)[d]	0.62[d]
With troponin T, LV ejection fraction, and proBNP ($n = 709$)				
Mortality	1.53 (1.36, 1.72)	<0.001	1.18 (1.01, 1.37)[e]	0.04[e]
Heart failure	1.34 (1.07, 1.36)	0.009	1.11 (0.85, 1.45)[e]	0.45[e]
Recurrent MI	1.22 (1.00, 1.49)	0.052	1.10 (0.86, 1.39)[e]	0.45[e]
Stroke	1.30 (0.95, 1.78)	0.10	1.01 (0.68, 1.48)[e]	0.97[e]

[a]HR, hazard ratio per 1 SD pg/mL increase in the natural logarithm of CgA.
[b]Adjusted for age, gender, index diagnosis, smoking status, prior MI, angina pectoris, diabetes, hypertension, congestive heart failure, heart rate, Killip class (>I) on admission, eGFR, and peak creatine kinase MB fraction.
[c]Adjusted for all variables listed in footnote b and troponin T.
[d]Adjusted for all variables listed in footnote b and troponin T and LV ejection fraction.
[e]Adjusted for all variables listed in footnote b and troponin T and LV ejection fraction and proBNP.

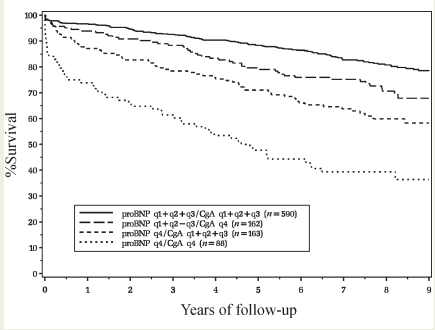

Figure 2 Association between chromogranin A (CgA) and pro-B-type natriuretic peptide (proBNP) levels by combined quartiles (*q*) and all-cause mortality in patients with acute coronary syndromes.

patients with echocardiographic data, the association was attenuated and no longer significant after adjustment for left ventricular ejection fraction. Potential reasons for the lack of a statistically significant independent association with heart failure in this subsample include the relative lack of statistical power and the fact that systolic dysfunction is a very strong predictor of heart failure. The association between CgA and recurrent MI was also attenuated after adjustment for conventional risk factors, but was borderline significant in patients in whom troponin T values were available.

In addition to its strong prognostic merit, several practical features make CgA a promising biomarker for clinical use, e.g. that its long *in vivo* half-life results in relatively high circulating concentrations. This feature simplifies blood collection and pre-analytic handling and makes CgA less prone to rapid fluctuations in circulating concentrations (low signal-to-noise ratio) than many other neurohormones.[17] Moreover, biochemical analysis of CgA can be readily performed using standardized and well-validated, commercially available assays.[22]

The two main causes of death in patients with ACSs are (i) recurrent ischaemic events, manifested as an ACS or sudden death, and (ii) heart failure, which may cause pulmonary congestion, inadequate tissue perfusion, or malignant arrhythmias. Although the univariable association between CgA and heart failure was closer than the associations between CgA and MI in the total cohort, in adjusted models the associations were of similar strength, permitting no clear conclusion to be drawn as to whether the prognostic value of CgA is mediated predominantly via prediction of heart failure or ischaemic events.

A potential link between the CgA and a propensity to heart failure development remains to be documented. However, theoretical considerations suggest that CgA is not only a marker of neuroendocrine activity, but may in itself exert harmful actions on the myocardium. CgA is a pro-hormone with multiple proteolytic cleavage sites,[4] allowing the generation of several peptides with different actions such as vasodilation,[23,24] negative inotropic actions,[25] inhibition of catecholamine secretion,[26] and induction of apoptosis.[27,28] Accordingly, some of the CgA-derived fragments could have effects of importance for cardiovascular homeostasis and the heart failure development, including catestatin, a potent non-competitive inhibitor of catecholamine release.[29] In a knock-out mouse model, obliteration of CgA gene expression resulted in decreased size and number of chromaffin granules as well as arterial hypertension and ventricular hypertrophy, whereas transgenic expression of human CgA and exogenous injection of human catestatin restored blood pressure.[30] These findings suggest that CgA and catestatin may play a significant role in cardiovascular homeostasis.

The stimulus for CgA production and the pathophysiological role CgA plays in ACSs remain to be accurately defined. Acute ischaemia and subsequent left ventricular dysfunction are both characterized by complex neuroendocrine and immune activation, and may both represent potential correlates of CgA production. Accordingly, the magnitude of the CgA response in ACSs may be related to the initial extent of myocardial injury and subsequent degree of ventricular dysfunction. It is also conceivable that CgA production is a compensatory response to the immune activation associated with ischaemia and heart failure development. Accordingly, in a mouse model, it has recently been demonstrated that CgA and its amino terminal fragments inhibit tumour necrosis factor α-induced increase in vascular permeability by preventing re-arrangement of the cytoskeleton,[31,32] suggesting that CgA could contribute to the regulation of endothelial barrier function.

The source of increased circulating levels of CgA in ACSs is not clear. CgA has been detected in the atrial secretory granules containing atrial natriuretic peptide,[33] and recently myocardial production of CgA in humans with dilated and hypertrophic cardiomyopathy has been demonstrated,[11] suggesting that CgA may be released from the myocardium in conditions characterized by pressure or volume overload. However, this does not rule out the possibility that other organs, including the adrenals, may be contributing sources to increased levels of CgA. Reduced clearance of CgA may also result in higher circulating levels.[34] Arterial and venous blood sampling across vascular beds will be required to determine organ-specific production and clearance of CgA.

Strengths and limitations

The prospective, observational design, long duration of follow-up, and, in a considerable proportion of patients, echocardiographic information concerning left ventricular systolic function and proBNP are all important strengths of the current single-centre study. In particular, objective measures of left ventricular systolic function are not commonly obtained or adjusted for in biomarker substudies of major pharmaceutical multi-centre trials in patients with ACSs. Limitations include the lack of troponin T, echocardiographic data, and/or proBNP in part of the patients, mainly because blood sampling was not performed systematically in the early phase of the study, and because echocardiography was not always feasible in patients who were discharged early. As data may not be missing completely at random, we cannot rule out the possibility of some extent of selection bias. However, given that the hazard ratio estimates do not vary widely between models, we believe that the bias is likely to be minor. Moreover, direct comparison of the hazard ratios of the different multivariate models in *Table 2*

should be avoided. There was relatively modest power to detect associations between CgA and specific morbidity endpoints. However, we believe that these limitations will tend to underestimate, rather than overestimate, the prognostic value of CgA.

Conclusions

This study shows that plasma CgA levels obtained within the first 24 h of admission are independently associated with the incidence of death in patients with ACS. Clinical use of CgA measurements for risk stratification purposes in patients with ACS must, however, await confirmatory evidence from other studies.

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Author contributions

A.M.J. interpreted the data and drafted the manuscript. H.R. contributed knowledge on chromogranin and drafted the manuscript in collaboration with A.M.J. T.O. participated in the design of the study and critically revised the paper. T.K. conducted the statistical analyses and critically revised the paper. M.H. conceived and designed the study and critically revised the paper. A.F. performed the CgA analyses and critically revised the paper. K.C. conceived and designed the study and critically revised the paper. K.C. and T.K. had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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CARDIOVASCULAR FLASHLIGHTS

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Aortoesophageal fistula, a catastrophic complication soon after successful repair of an aortic dissection type A

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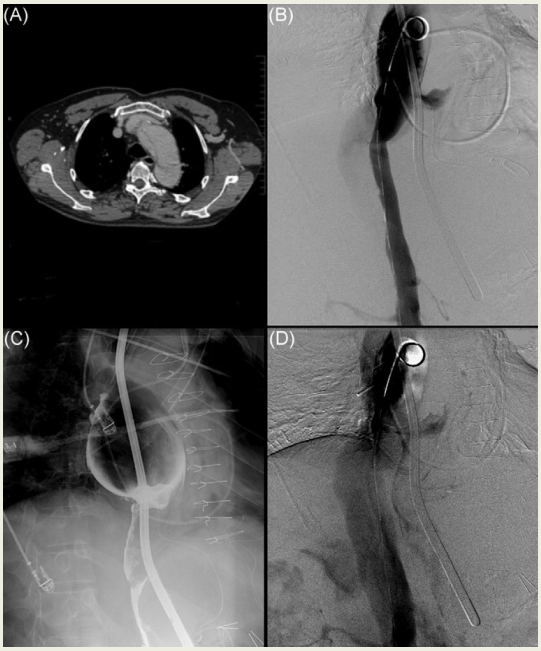
A 66-year-old man with a history of arterial hypertension underwent emergency cardiac surgery for aortic dissection type A, diagnosed by a computed tomographic scan (*Panel A*) after abdominal pain and syncope. The lesion was repaired with a Dacron tubular prosthesis. In the postoperative period, he improved his condition slowly under mechanical ventilation and inotropic support. At postoperative day 12, he was awake with minimum inotropic support and in weaning from mechanical ventilation.

Suddenly, he presented massive haematemesis. Under the suspicion of an aorto-oesophageal fistula, an urgent upper gastrointestinal endoscopy was performed, showing active bleeding at 36 cm from the dental arcade.

Resuscitation required transfusion of 13 packed red blood cells, four units of fresh frozen plasma, and seven units of platelets.

A Sengstaken–Blackmore tube was inserted in order to contain the bleeding, which was successful for a while.

After stabilization, an aortogram was performed (*Panels B–D*), which revealed contrast leak with active bleeding in the descending thoracic aorta from the true lumen to the oesophagus, at the level of the gastric balloon of the Sengstaken tube which was placed at the oesophagus (*Panel C*).



The placement of an endovascular stent graft was impossible because of the extensive lesions in the aortic wall.

The patient died 15 h after the initial bleeding, in a situation of refractory shock and persistent bleeding.

Aortoesophageal fistula is an uncommon complication in the early postoperative period of aortic dissection type A, usually fatal as a result of exsanguinating haemorrhage before assessment and any treatment can be undertaken.

II

III

IV

Secretogranin II; a protein increased in the myocardium and circulation in heart failure with cardioprotective properties

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Short title: Secretogranin II in heart failure

Abstract: 258 Text: 3289 Tables: 1 Figures: 7

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ABSTRACT

Background: Several beneficial effects have been demonstrated for secretogranin II (SgII) in non-cardiac tissue. As cardiac production of chromogranin A and B, two related proteins, is increased in heart failure (HF), we hypothesized that SgII could play a role in cardiovascular pathophysiology.

Methodology/Principal findings: SgII production was characterized in a post-myocardial infarction heart failure (HF) mouse model, functional properties explored in experimental models, and circulating levels measured in patients with stable HF of moderate severity. SgII mRNA levels were 11.5 fold upregulated in the left ventricle (LV) of animals with myocardial infarction and HF ($p<0.001$ vs. sham-operated animals). SgII protein levels were also increased in the LV, but not in other organs investigated. SgII production was confined to cardiomyocytes, and potently induced by transforming growth factor-β and norepinephrine stimulation *in vitro*. Processing of SgII to shorter peptides was enhanced in the failing myocardium due to increased levels of the proteases PC1/3 and PC2. Examining a pathophysiological role of SgII in myocardial infarction and HF, the SgII fragment secretoneurin reduced myocardial ischemia-reperfusion injury and cardiomyocyte apoptosis by 30% and rapidly increased cardiomyocyte Erk1/2 and Stat3 phosphorylation. Circulating levels of SgII were higher in HF patients than in control subjects: 0.16 (Q1-3 0.14-0.18) vs. 0.12 (0.10-0.14) nmol/L, $p<0.001$.

Conclusions: We demonstrate increased myocardial SgII production and processing in the LV in animals with myocardial infarction and HF, which could be beneficial as the SgII fragment secretoneurin protects from ischemia-reperfusion injury and cardiomyocyte apoptosis. Circulating SgII levels are also increased in patients with HF and may represent a new cardiac biomarker.

Key words: Heart failure, molecular biology, ischemia, secretogranin II, secretoneurin, biological markers

INTRODUCTION

Heart failure (HF) is associated with changes in systemic and pulmonary hemodynamics, complex neurohumoral activation, as well as local molecular alterations in the myocardium [1]. Given that coronary artery disease is a leading cause of HF [1], a better understanding of the pathophysiology of myocardial ischemia and HF development is needed. Regulation of hormonal factors in the failing myocardium can directly affect function and survival of cardiac cells [1], and result in increased circulating levels of proteins secreted from the myocardium [2]. Thus, by identifying proteins that are regulated in the failing myocardium, we may enhance our understanding of the pathophysiology of HF and discover new diagnostic and prognostic HF biomarkers.

The protein secretogranin II (SgII) is a 587 amino acid long protein from the chromogranin-secretogranin (granin) protein family [3]. Two other members of the granin protein family, chromogranin (Cg) A and B have been found increased in HF [4-6], and may represent novel cardiac biomarkers [4-9]. In addition, chromogranins may affect myocardial function in HF [10,11]. For SgII, functional aspects have mainly been attributed to the short 33 amino acid peptide secretoneurin ($SgII_{154-186}$) and a number of interesting pathophysiological effects have been reported in other organs than the heart [3]. SgII production is increased by hypoxia in skeletal muscle [12], and secretoneurin protects against apoptosis and ischemic injury in the brain and skeletal muscle [13,14]. The proteases PC1/3 and PC2 have been identified as the principal proteases for processing of SgII to shorter fragments [15,16]. As other granin proteins appear to be upregulated during HF development, we hypothesized that SgII production is increased in the myocardium and circulation in HF, and that SgII could play a role in the pathophysiology of HF following myocardial ischemia.

RESULTS

Left ventricular SgII gene expression is upregulated during HF development

To study SgII production in the left ventricle (LV) in HF, we first compared SgII mRNA levels in non-infarcted LV tissue of HF animals to levels in sham animals. HF animals exhibited increased lung weights and increased LV and right ventricular mass, reflecting pulmonary congestion and compensatory myocardial hypertrophy (Supplementary Table 1). LV SgII mRNA levels were markedly upregulated in HF animals compared to sham animals (11.5 fold increase, $p<0.001$, Figure 1A). This was a greater relative increase than observed for B-type natriuretic peptide (BNP), CgA, or CgB mRNA levels (Supplementary Table 1).

SgII mRNA levels correlated significantly with CgA mRNA levels in HF (r=0.68, p=0.04, Figure 1B), but not with CgB or BNP mRNA levels (Supplementary Table 2).

SgII is produced by cardiomyocytes and increase in the LV in HF

In parallel with observed alterations in SgII mRNA expression, protein levels of SgII were significantly increased in both the non-infarcted (35% increase, p=0.02) and the infarcted region of the LV (85% increase, p<0.001) in HF animals as measured by radioimmunoassay (RIA) (Figure 2A). As previously reported for chromogranins [6,17], SgII immunoreactivity in the myocardium was solely confined to cardiomyocytes (Figure 2B). The complex processing of SgII (Figure 3A) [3,18] was examined by immunoblotting, and we identified increased processing of SgII to shorter SgII fragments in the LV of HF animals compared to sham animals (Figure 3B). Finally, levels of the SgII protease PC1/3 were potently increased in the non-infarcted and infarcted region of the LV of HF animals (350% increase vs. sham in both, p<0.001), while the active form of the SgII protease PC2 (68 kDa) [19] was only increased in the infarcted region of HF animals (110% increase, p=0.02) (Figure 3C).

SgII production was not increased in other tissues investigated

To further study SgII production in HF, we measured SgII protein levels in non-cardiac tissue by RIA. As illustrated in Figure 4, SgII levels were not altered in the right ventricle, liver, spleen, kidney, stomach, colon, and skeletal muscle during HF development, while SgII levels were decreased by 19% (p=0.03) in pulmonary tissue of HF animals. Processing of SgII to shorter fragments was not enhanced in HF outside of the LV (Figure 3B).

Transforming growth factor-β and norepinehrine increase SgII gene expression in cardiomyocytes

As multiple endocrine and paracrine factors are known to influence cardiomyocyte protein synthesis [1], we examined possible factors that could influence SgII production in cardiomyocytes. Stimulating neonatal rat cardiomyocytes *in vitro* for 24 h with transforming growth factor-β (TGF-β) and norepinephrine (NE) increased SgII mRNA levels by 85% (p<0.001) and 35% (p=0.02), respectively, while exposure to angiotensin II (AngII), endothelin-1 (ET-1), and tumor necrosis factor-α (TNF-α) did not affect SgII production (Figure 5). Forskolin (FSK), which has been shown to induce SgII mRNA expression in other cell types [20], was used as a positive control and increased SgII mRNA levels in isolated cardiomyocytes by 35% (p=0.001).

The SgII fragment secretoneurin reduces ischemia/reperfusion injury in the isolated perfused rat heart

As SgII production was increased in the infarcted LV, we assessed whether the SgII fragment secretoneurin could protect against damage after myocardial ischemia. In a global ischemia/reperfusion (I/R) model of the isolated perfused rat heart, the addition of secretoneurin to the buffer reduced infarct size by 30% (p=0.047) after 30 min of ischemia and 2 h of reperfusion (Figure 6A). The beneficial effects of secretoneurin on I/R damage were also evidenced by lower LV end-diastolic pressure in the hearts perfused with secretoneurin (Figure 6A).

Secretoneurin stimulation increases Stat3 and Erk1/2 phosphorylation and reduces hydrogen peroxide-induced apoptosis in isolated cardiomyocytes

Reduced apoptosis in cardiomyocytes at risk could be a mechanism by which secretoneurin protects the myocardium after I/R injury. We therefore investigated whether secretoneurin plays a role in the protection of cardiomyocytes exposed to hydrogen peroxide (H_2O_2). Exposure of H_2O_2 to cardiomyocytes markedly induced apoptosis, as indicated by measurements of DNA fragmentation (Figure 6B). We observed that the proportion of apoptotic cells after H_2O_2 exposure was 30% lower (p=0.005) in cells co-stimulated with secretoneurin compared to cells in standard medium. Finally, we examined signaling pathways that could account for the effects of secretoneurin on I/R injury and cardiomyocyte apoptosis. Phospho Akt_{Ser473}, $Erk1/2_{Thr202/Tyr204}$, and $Stat3_{Ser727/Tyr705}$ levels were measured in cardiomyocytes after short-term secretoneurin exposure. Secretoneurin stimulation significantly increased $Erk1/2_{Thr202/Tyr204}$ phosphorylation (four and two fold increase after 10 and 30 min, respectively, p=0.04) (Figure 6C). $Stat3_{Ser727}$ phosphorylation was increased after 10 min of secretoneurin exposure (100% increase, p=0.04), while phospho $Stat3_{Tyr705}$ and Akt_{Ser473} levels were unaltered.

Circulating levels of SgII are increased in patients with stable HF

To examine SgII production in patients, we compared plasma levels of SgII in 58 HF patients and 20 age- and gender-matched healthy control subjects (Table). In this cohort of stable HF patients treated according to updated guidelines, SgII levels were higher than levels in the control group: median 0.16 (Q1-3 0.14-0.18) vs. 0.12 (0.10-0.14) nmol/L, p<0.001 (Figure 7). Plasma levels of SgII correlated with BNP in HF patients (r=0.26, p=0.05), but not in control subjects (r=0.19, p=0.43). SgII levels did not correlate with CgA or CgB levels in control

subjects or HF patients (Supplementary Table 3). As evaluated by receiver operating analysis, circulating levels of SgII were more closely regulated in HF than both CgA (AUC=0.84 for SgII vs. AUC=0.57 for CgA, p=0.001) and CgB levels (AUC=0.68 for CgB, p=0.03 vs. SgII). Use of a proton pump inhibitor (PPI), a medication known to increase circulating CgA levels [6], did not increase SgII levels as levels in patients using PPI (n=8) were similar to SgII levels in the other HF patients: 0.16 (Q1-3 0.13-0.19) vs. 0.16 (0.14-0.18) nmol/L, respectively, p=0.68).

DISCUSSION

In the present study we demonstrate for the first time that the expression of SgII, a member of the granin protein family, is increased in the LV during HF development. Moreover, SgII processing to shorter fragments is increased in the LV in post-infarction HF, which could be beneficial as the SgII fragment secretoneurin protects against myocardial I/R injury and cardiomyocyte apoptosis. SgII production was not increased in other tissues investigated, and therefore the LV could be a significant contributor to the elevated circulating SgII levels in patients with HF.

Myocardial production of SgII in HF

HF is associated with increased myocardial production of several protein families [1,2]. Like other granin proteins, SgII is a pro-hormone with multiple dibasic cleavage sites [3]. Although the processing of SgII is known to be more complex than for most other pro-hormones [3,21], SgII has in several tissues been demonstrated to be almost fully processed to the short 33 amino acid peptide secretoneurin [22]. In the circulation, the majority of SgII immunoractivity is also reportedly found in the form of secretoneurin [22]. Pertinent to this, data from non-cardiac tissue have indicated that cells with an enhanced secretory rate exhibit increased processing of SgII to shorter fragments [18]. Accordingly, our novel data demonstrating increased levels and processing of SgII to shorter fragments in the LV during post-myocardial infarction HF point to the myocardium as a significant contributor to the increased circulating levels of SgII in HF. This is supported by the lack of increase in SgII production in non-cardiac tissue in HF. The mechanism by which SgII processing is increased seems to be enhanced PC1/3 (previously denoted PC1 or PC3) and PC2 activity in the LV, which are the principal proteases of SgII [15,16]. The complex processing of SgII in the LV with several shorter fragments corresponds to immunoblots of SgII processing in non-cardiac tissue [15,16,18,21,22]. One report has previously demonstrated increased PC1/3 mRNA

levels close to the infarct area in rats with myocardial infarction and HF [23], but to our knowledge the increase in both PC1/3 and PC2 in the LV of animals with HF has previously not been reported. Increased PC1/3 and PC2 activity has been hypothesized as a conceptual model for enhanced SgII processing in non-cardiac cells [18].

We identified cardiomyocytes as the cell type responsible for SgII production in the myocardium. This is in line with previous data for chromogranins [6,17], and with a report published during progression of our work that demonstrates the presence of SgII in the healthy rodent myocardium [24]. We have also previously demonstrated that NE, TGF-β, and AngII regulate CgB production in cardiomyocytes [6]. Similar to this, we now report that TGF-β and NE both induce SgII mRNA expression in cardiomyocytes *in vitro*, while AngII failed to increase SgII mRNA levels. Distinct regulation of different granins has been reported in other cell types and may be explained by variations in the promoter region, including the serum response element (SRE), which is only found in the SgII promoter [20]. Effect of growth factors on SRE promoter sites could explain the potency of TGF-β as a stimulus for SgII production in cardiomyocytes [20], but the precise mechanism regulating cardiomyocyte SgII production needs to be established in future studies. This also relates to a possible role of hypoxia-inducible factor-1α (HIF-1α) as a regulator of SgII production after myocardial ischemia. Indeed, HIF-1α has previously been found to increase SgII production in skeletal muscle subjected to hypoxia [12], and we found SgII production increased in the LV of animals with myocardial infarction and HF.

Functional aspects of the SgII fragment secretoneurin in the myocardium

Proteins produced by the heart during the development of HF may exert their actions via endocrine, paracrine or autocrine mechanisms [1]. Findings in the current study suggest that SgII and the fragment secretoneurin could exert important effects in the myocardium by reducing I/R injury and cardiomyocyte apoptosis. These effects of secretoneurin, together with the observation that LV SgII levels and processing are increased in animals with myocardial infarction and HF, are compatible with known functional properties of secretoneurin. Secretoneurin has previously been found to attract leukocytes, especially monocytes [25], and may be of importance for the early post-infarction inflammatory response in the myocardium. Secretoneurin also attracts endothelial cells, both mature endothelial cells for angiogenesis [26] and bone marrow-derived progenitor endothelial cells

for vasculogenesis [27], suggesting that secretoneurin could play a role in revascularization of infarcted myocardial tissue.

In this study, we have shown for the first time that secretoneurin attenuates cardiomyocyte apoptosis and reduces infarct size in the subacute phase after myocardial infarction. A putative mechanism for this effect of secretoneurin against I/R injury, is via reduction of cardiomyocyte apoptosis in the border zone, possibly mediated by Erk1/2 and Stat3 activation [28,29]. Other studies have shown that secretoneurin protects endothelial cells from apoptosis through Erk1/2 signaling [26,27], and attenuates neuronal cell death after ischemia by induction of the Jak/Stat pathway [13], providing support for secretoneurin as a protective factor in the subacute phase of tissue ischemia. However, we have not assessed the long-term effects of increased SgII levels for organ function in HF and this should be explored in future studies. Before the long-term effects of secretoneurin have been established, the net effect by increased SgII levels in HF cannot be estimated, although we provide evidence that LV SgII production seems to be protective in the subacute phase of post-infarction HF development.

Circulating SgII levels in patients with HF

In this first report on SgII in HF, we find circulating SgII levels to be increased in stable patients with mainly moderate severity of HF compared to age- and gender-matched control subjects. We also find SgII levels to be more closely increased in our patients with stable HF compared to CgA and CgB levels, but whether SgII have additional diagnostic and prognostic utility over other granin biomarkers in HF will need to be explored in future studies.

Conclusion

We have observed increased myocardial SgII production and processing to shorter fragments in animals with myocardial infarction and HF. This could be beneficial as the SgII fragment secretoneurin protects from I/R injury and cardiomyocyte apoptosis. As SgII production was not enhanced in other tissues investigated, LV SgII production could be a significant contributor to the elevated circulating levels of SgII in patients with HF.

METHODS

Animal experiments were performed according to recommendations from the European Council for Laboratory Animal Science and approved by the Norwegian Council for Animal Research (#HR0506). The study protocol of the clinical study was approved by the South-Eastern Regional Ethics Committee Norway (#1.2006.2653) before the initiation of the study, and all participants gave their written informed consent prior to study commencement.

Mouse model of HF

Mice were anesthetized with 0.2 mg propofol in the tail vein, trachetomized, connected to an animal ventilator, and ventilated with a mixture of 98% oxygen and 2% isoflurane during surgery. A permanent ligation of the left main coronary artery was performed in mice and the animals were evaluated by echocardiography for development of HF [30]. Sham-operated (sham) animals underwent the same procedure without coronary artery ligation. All efforts were made to minimize suffering. Animals were sacrificed at one week post-surgery, when hearts and other organs were dissected, prepared, and stored as previously described [6].

RT-qPCR, radioimmunoassays, immunhistochemistry, and immunoblotting

mRNA levels were measured with TaqMan Gene Expression assays from Applied Biosystems (Foster City, CA, USA): (i) mouse myocardium: SgII (Mm00843883_s1), CgA (Mm00514341_m1), CgB (Mm00483287_m1), BNP (Mm00435304_g1), ribosomal protein L4 (RPL4) (Mm00834993_g1); and (ii) neonatal rat cardiomyocytes: SgII (Rn01400686_g1) and RPL4 (Rn00821091_g1). SgII protein levels in tissue and plasma were measured by a RIA binding to the secretoneurin region of SgII ($SgII_{154-165}$) [31]. The detection limit of the SgII RIA in plasma is 0.05 nmol/L and the assay has a coefficient of variation (CV) of 9% in the lower range (1.10 nmol/L) and 4% in the upper range (3.80 nmol/L). We used a commercial RIA for CgA analysis (EuroDiagnostica AB, Malmö, Sweden) with a detection limit of 0.8 nmol/L and a CV of 13% in the lower range (3.1 nmol/L) and 9% in the upper range (17.0 nmol/L), while an in-house RIA was used for CgB analysis with detection limit ≥0.80 nmol/L and a CV of 17% in the lower range (1.40 nmol/L) and 8% in the upper range (6.40 nmol/L) [32]. For immunoblotting we used an N-terminal SgII antibody (ab20246, Abcam, Cambridge, UK) as SgII processing has been reported to start from the C-terminal end [18]. We quantitated three bands of SgII (81, 66, and 55 kDa) according to previous data on SgII processing in non-cardiac tissue [18]. Additional antibodies for immunoblotting and immunhistochemistry are reported in the Data Supplement.

Regulation of SgII mRNA production in neonatal rat cardiomyocytes

After 24 h starvation of the cells, neonatal rat cardiomyocytes were stimulated as previously reported [6] for 24 h with the following agents; forskolin (FSK) [10 μM], norepinephrine (NE) [100 μM], endothelin-1 (ET-1) [250 ng/mL], angiotensin II (AngII) [1 μM] (all Sigma-Aldrich, St. Louis, MO, USA), tumor necrosis factor-α (TNF-α) [10 ng/mL] (BioSource International, Camarillo, CA, USA), and transforming growth factor-β (TGF-β) [10 ng/mL] (R&D Systems, Minneapolis, MN, USA).

Langendorff perfusion

All rats were anesthetized with 5% sodium pentobarbital (60-80 mg/kg intraperitoneally (i.p)) and heparinised (500 IU i.p). After anesthesia, rat hearts were rapidly excised and placed in ice-cold Krebs-Henseleit Buffer (KHB) (mmol/L: NaCl 118.5; $NaHCO_3$ 25; KCl 4.7; KH_2PO_4 1.2; $MgSO_4/7H_2O$ 1.2; glucose/$1H_2O$ 11.1; $CaCl_2$ 1.8) for further dissection. After aortic cannulation, the hearts were mounted on a Langendorff system (AD Instruments Pty Ltd, Castle Hill, NSW 2154, Australia) and retrogradely perfused with warm (37ºC), oxygenated (95% O_2, 5% CO_2) KHB at constant pressure of 70 mmHg. After 40 min of stabilization, hearts were subjected to 30 min of global ischemia, and then reperfused for 120 min (Supplementary Figure). In the experimental group (secretoneurin group), 0.66 μg/mL secretoneurin (NeoMPS, Strasbourg, France) was added to the perfusate 20 min prior to ischemia, and was also used throughout the reperfusion period. A recirculation system was inserted from 20 min prior to induction of ischemia in both groups (Supplementary Figure), and used throughout the reperfusion period. Infarct size was calculated with Adobe Photoshop (Adobe Systems, San Jose, CA, USA) as percentage of the total area by an investigator blinded to treatment.

Effect of secretoneurin on phospho proteins

To assess the short-term effect of secretoneurin stimulation on phospho proteins, the cardiomyocytes were stimulated for 10 or 30 min with 10 μg/mL secretoneurin (NeoMPS) or vehicle. Cells were harvested in lysis buffer (Tris pH 7.6, 5M NaCl, 0.5M EDTA, 0.1M EGTA, 1M B-gly [Sigma-Aldrich St. Louis, MO, USA] and NP-40), and the protein levels measured by immunoblotting (details reported in the Data Supplement).

Cell Death Enzyme-linked Immunosorbent Assay

Neonatal rat cardiomyocytes were stimulated with 10 μg/mL secretoneurin (NeoMPS) or vehicle for 24 h, followed by 24 h exposure to hydrogen peroxide (H_2O_2, 100 μmol) in the presence of secretoneurin or vehicle. Cells were then harvested in lysis buffer and apoptosis assessed using the Cell Death Enzyme-linked Immunosorbent Assay (Cell Death Detection ELISA^{PLUS}, Roche, Basel, Switzerland) according to manufacturer procedure.

Patients with stable HF and control subjects

Fifty-eight patients with echocardiographically documented impaired systolic function [LV ejection fraction (LVEF) ≤50%], and no hospitalization for HF during the last three months prior to study commencement, were compared to 20 control subjects with no history or current symptoms of chronic disease. The control subjects had a normal physical examination, did not use medication regularly, and were hospital employees or recruited from outside of the hospital.

Statistical analysis

Continuous data are presented as mean (± SEM) or median (quartile [Q] 1-3) and categorical values as counts (percentage). Continuous variables were compared by Student's *t*-test except circulating biomarker values that were compared by the Mann-Whitney U-test due to a right-skewed distribution. Serial data were compared by Two-Way ANOVA, and categorical data by the chi-square test or Fisher's exact test as appropriate. Correlations were calculated by Spearman rank correlation. P-values <0.05 were considered significant for all analyses. Statistical analyses were performed with SPSS for Windows version 16.0 (SPSS, Chicago, IL) except receiver operating analysis which was performed with MedCalc for Windows, version 9.5.1.0 (MedCalc Software, Mariakerke, Belgium) by the method of Hanley and McNeil [33].

Details regarding methods can be found in the Data Supplement.

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DISCLOSURES

We have the following interest. H.R., M.S., T.O, and G.C. are partners in a patent application filed by the University of Oslo regarding the use of SgII as a cardiac biomarker.

AUTHOR CONTRIBUTION

Conceived and designed the experiments: HR, TO, GC. Performed the experiments: HR, MS, GF, KOS, AHO, IS, CH, MBD, EØ, WEL. Analyzed the data: HR, MS, GF, KOS, AHO, IS, CH, MBD, EØ, WEL, TO, GC. Wrote the paper: HR, TO, GC

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FIGURE LEGENDS

Figure 1. Left ventricular SgII gene expression in heart failure. A, SgII mRNA levels in non-infarcted left ventricular tissue during HF development. SgII mRNA levels were 11.5 fold increased ($p<0.001$) in non-infarcted LV tissue in HF animals (n=9) compared to sham-operated animals (n=8). Gene expression was measured by RT-qPCR and is presented as fold change ± SEM. B, LV SgII mRNA levels were closely correlated with CgA mRNA levels in both HF ($r=0.68$, $p=0.04$) and sham animals ($r=0.81$, $p=0.02$).
** $p<0.001$

Figure 2. SgII is produced by cardiomyocytes and increased in the left ventricle during HF development. A, SgII protein levels as measured by RIA were increased in both the non-infarcted and infarcted region of the LV in HF animals compared to levels in the myocardium of sham-operated animals (n=9 for both groups). B, Representative photomicrographs of myocardial tissue sections of a HF mouse demonstrating SgII immunoreactivity (brown staining) in cardiomyocytes bordering the infarcted zone (border zone, lower left), in the remote non-infarcted myocardium (upper left), and in surviving cardiomyocytes of the infarcted region (upper right). Bottom right picture demonstrates very weak staining after use of non-immune rabbit serum as control (ctr). Magnification: ×200.
** $p<0.001$, * $p<0.01$.

Figure 3. SgII processing is increased in the left ventricle of HF mice. A, Figure of SgII processing as reported in non-cardiac tissue (modified from ref. 18). B, SgII processing in the left and right ventricles in HF and sham animals. Bands at 81 kDa, 66 kDa, and 55 kDa were measured and are presented as fold change (SEM) vs. sham animals (n=6 for each group). C, Levels of the proteases PC1/3 and PC2 in the left and right ventricles in HF and sham animals.

Figure 4. SgII production outside of the left ventricle in heart failure. SgII levels were decreased in pulmonary tissue during HF development, while levels were unchanged in the other tissues examined. SgII levels in the (A) right ventricle, (B) pulmonary tissue, (C) liver, (D) spleen, (E) kidney, (F) stomach, (G) colon, and (H) skeletal muscle were measured by RIA and are presented as fold change ± SEM (n=6 for both groups, except pulmonary tissue: HF: n=14, sham: n=13).
# $p<0.05$.

Figure 5. Regulation of cardiomyocyte SgII expression by important hormonal and paracrine factors in HF. SgII mRNA levels were measured by RT-qPCR after stimulating neonatal rat cardiomyocytes for 24 h with either PBS (Ctr, n=9), forskolin (FSK n=5), norepinephrine (NE, n=5), angiotensin II (AngII, n=4), endothelin-1 (ET-1, n=5), transforming growth factor-β (TGF-β, n=6), or tumor necrosis factor-α (TNF-α, n=6). SgII mRNA levels are presented as fold change ± SEM vs. PBS-stimulated cells.
** p<0.001, * p<0.01, # p<0.05.

Figure 6. The secretogranin II fragment secretoneurin has protective effects during myocardial ischemia and cardiomyocyte stress. A, Secretoneurin reduces infarct size by 30% (upper left) and improves myocardial function (upper right) after global ischemia in the isolated perfused rat heart. B, Cardiomyocyte apoptosis *in vitro* after H_2O_2 exposure was attenuated by secretoneurin stimulation. Cells were extracted from 5 different cell isolations (n=5 for all groups). C, Short-term stimulation of cardiomyocytes with 10 µg/mL secretoneurin (SN) activated protective intracellular pathways as reflected by increased Stat3 and Erk1/2 phosphorylation (n=5 for all groups).
** p<0.001, * p<0.01, # p<0.05.

Figure 7. Circulating SgII levels are elevated in patients with chronic, stable HF. SgII levels were significantly increased in HF patients (n=58) compared to healthy age- and gender-matched control subjects (n=20): Median 0.16 (Q1-3 0.14-0.18) vs. 0.12 (0.10-0.14) nmol/L, p<0.001. HF patients are presented according to NYHA functional class. The horizontal line within the box represents the median level, the boundaries of the box the 25th and 75th percentile levels, and the whiskers the 10th-90th percentile.
** p<0.001

Table. Descriptive statistics of heart failure patients and control subjects

	Control subjects (n=20)	HF patients (n=58)	p
Male sex (n, %)	16 (80%)	47 (81%)	0.92
Age, years (mean ± SEM)	60.6 ± 1.1	62.8 ± 1.6	0.43
NYHA class (n, %)			
II		47 (81%)	
III		11 (19%)	
Etiology for HF (n, %)			
Ischemic		35 (60%)	
Dilated cardiomyopathy		21 (36%)	
Other		2 (4%)	
Duration of HF, months (median, Q1-3)		18 (12-48)	
LVEF, % (mean ± SEM)		33 ± 1	
Medication (n, %)			
β-blocker		57 (98%)	
ACEI		41 (71%)	
ARB		17 (29%)	
ACEI or ARB		58 (100%)	
Aldosterone antagonist		11 (19%)	
Diuretic		43 (74%)	
Statin		38 (65%)	
Warfarin		29 (50%)	
ASA		34 (59%)	
Clopidogrel		7 (12%)	
Digitalis		14 (24%)	
Amiodarone		6 (10%)	
Nitrate		6 (10%)	
PPI		8 (14%)	
CRT		10 (17%)	
ICD		11 (19%)	
CgA levels, nmol/L	4.5 (4.0-5.3)	5.0 (3.5-8.2)	0.33
CgB levels, nmol/L	1.47 (1.39-1.58)	1.63 (1.44-1.80)	0.02

BNP levels, pg/mL	26 (13-37)	197 (89-338)	<0.001

NYHA class indicates New York Heart Association functional class; Q1-3, quartile 1-3; LVEF, left ventricular ejection fraction; ACEI, angiotensin converting enzyme inhibitor; ARB, angiotensin II receptor blocker; ASA, acetyl salicylic acid; PPI, proton pump inhibitor; CRT, cardiac resynchronization therapy; ICD, implantable cardioverter-defibrillator; CgA, chromogranin A; CgB, chromogranin B; and BNP, B-type natriuretic peptide. Biomarker levels are presented as median (quartile 1-3).

Figure 1.

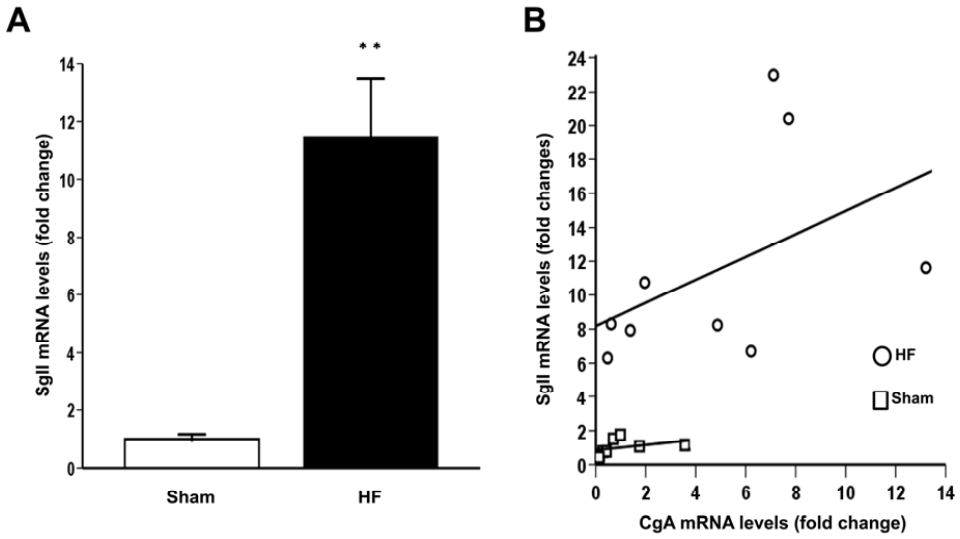

A
B
SgII mRNA levels (fold change)
Sham
HF
SgII mRNA levels (fold changes)
CgA mRNA levels (fold change)
HF
Sham

Figure 2.

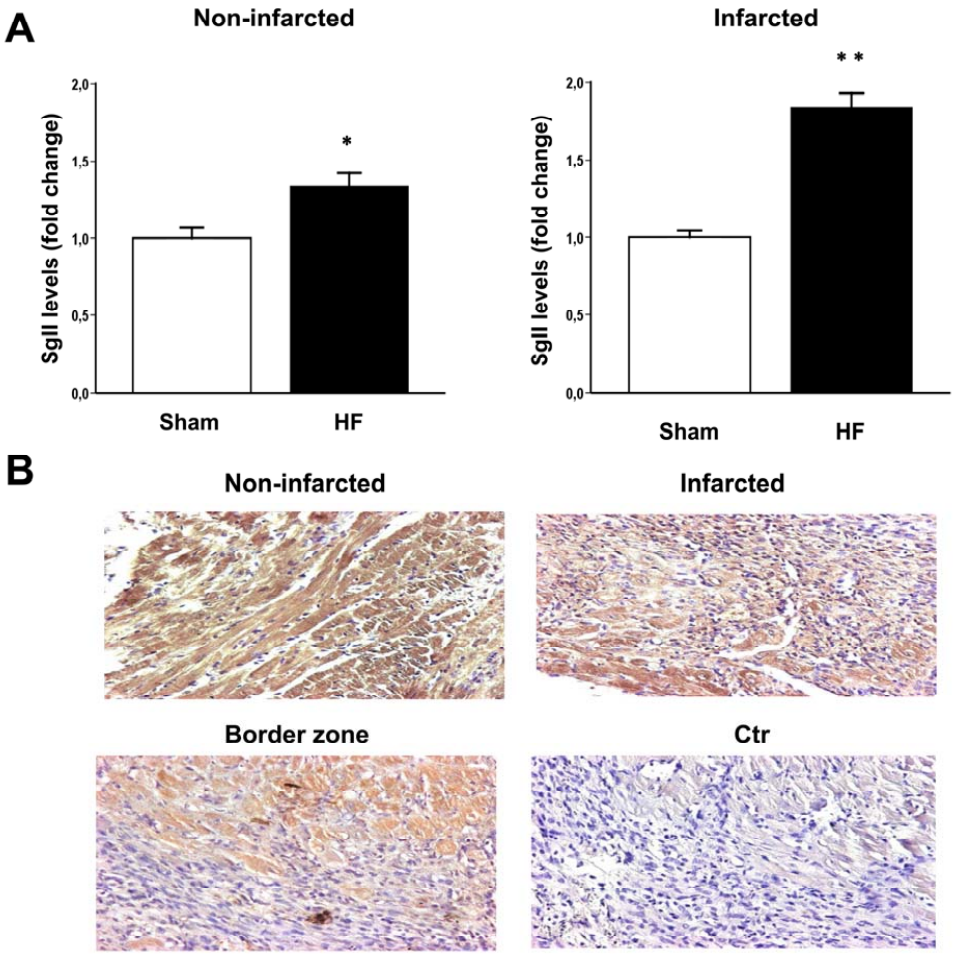

A
Non-infarcted
Infarcted
SgII levels (fold change)
2,0
1,5
1,0
0,5
0,0
*
* *
Sham
HF
B
Non-infarcted
Infarcted
Border zone
Ctr

Figure 3.

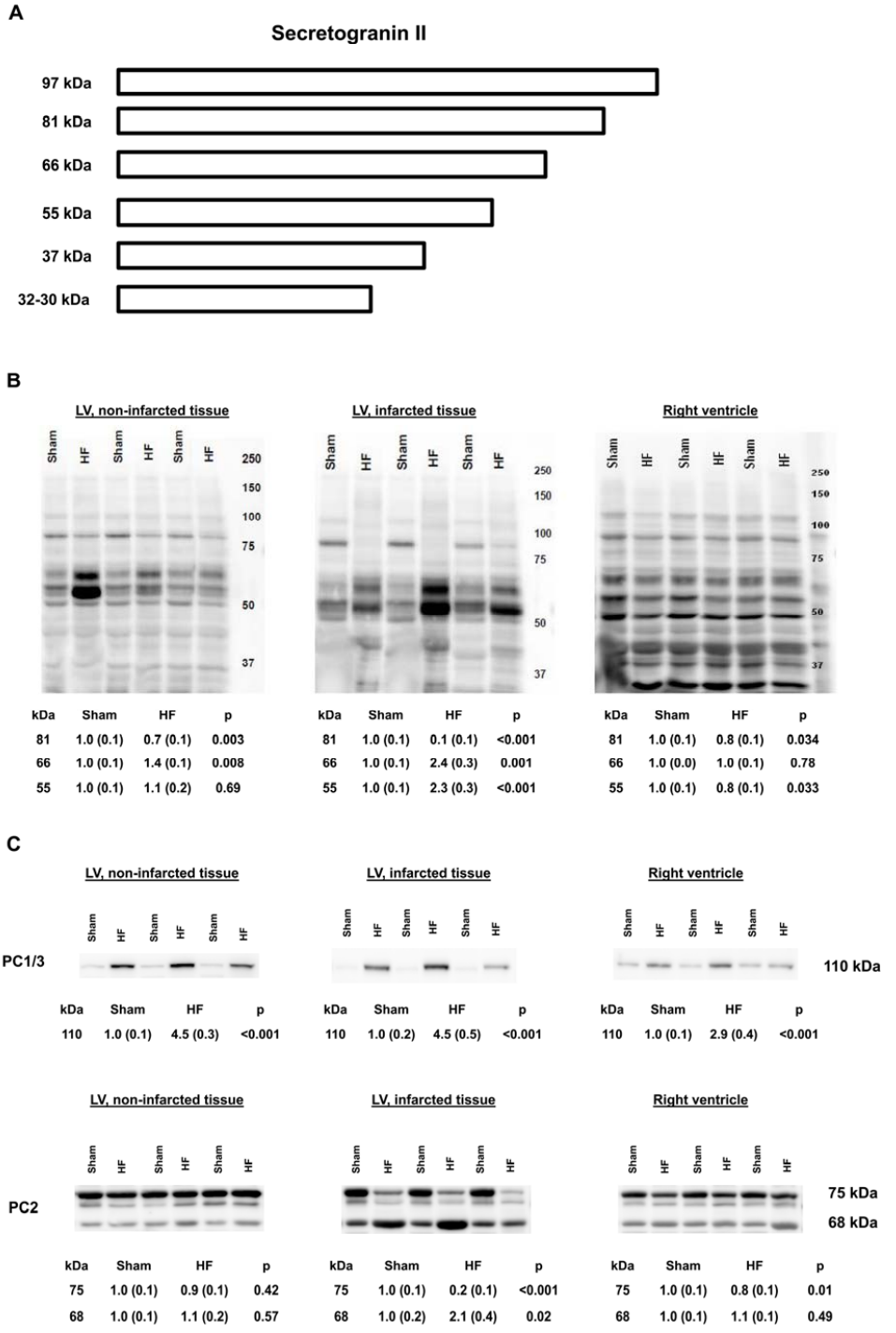

A
Secretogranin II
97 kDa
81 kDa
66 kDa
55 kDa
37 kDa
32-30 kDa
B
LV, non-infarcted tissue
LV, infarcted tissue
Right ventricle
Sham
HF
250
150
100
75
50
37
kDa Sham HF p
81 1.0 (0.1) 0.7 (0.1) 0.003
66 1.0 (0.1) 1.4 (0.1) 0.008
55 1.0 (0.1) 1.1 (0.2) 0.69
kDa Sham HF p
81 1.0 (0.1) 0.1 (0.1) <0.001
66 1.0 (0.1) 2.4 (0.3) 0.001
55 1.0 (0.1) 2.3 (0.3) <0.001
kDa Sham HF p
81 1.0 (0.1) 0.8 (0.1) 0.034
66 1.0 (0.0) 1.0 (0.1) 0.78
55 1.0 (0.1) 0.8 (0.1) 0.033
C
LV, non-infarcted tissue
LV, infarcted tissue
Right ventricle
PC1/3
110 kDa
kDa Sham HF p
110 1.0 (0.1) 4.5 (0.3) <0.001
kDa Sham HF p
110 1.0 (0.2) 4.5 (0.5) <0.001
kDa Sham HF p
110 1.0 (0.1) 2.9 (0.4) <0.001
LV, non-infarcted tissue
LV, infarcted tissue
Right ventricle
PC2
75 kDa
68 kDa
kDa Sham HF p
75 1.0 (0.1) 0.9 (0.1) 0.42
68 1.0 (0.1) 1.1 (0.2) 0.57
kDa Sham HF p
75 1.0 (0.1) 0.2 (0.1) <0.001
68 1.0 (0.2) 2.1 (0.4) 0.02
kDa Sham HF p
75 1.0 (0.1) 0.8 (0.1) 0.01
68 1.0 (0.1) 1.1 (0.1) 0.49

Figure 4.

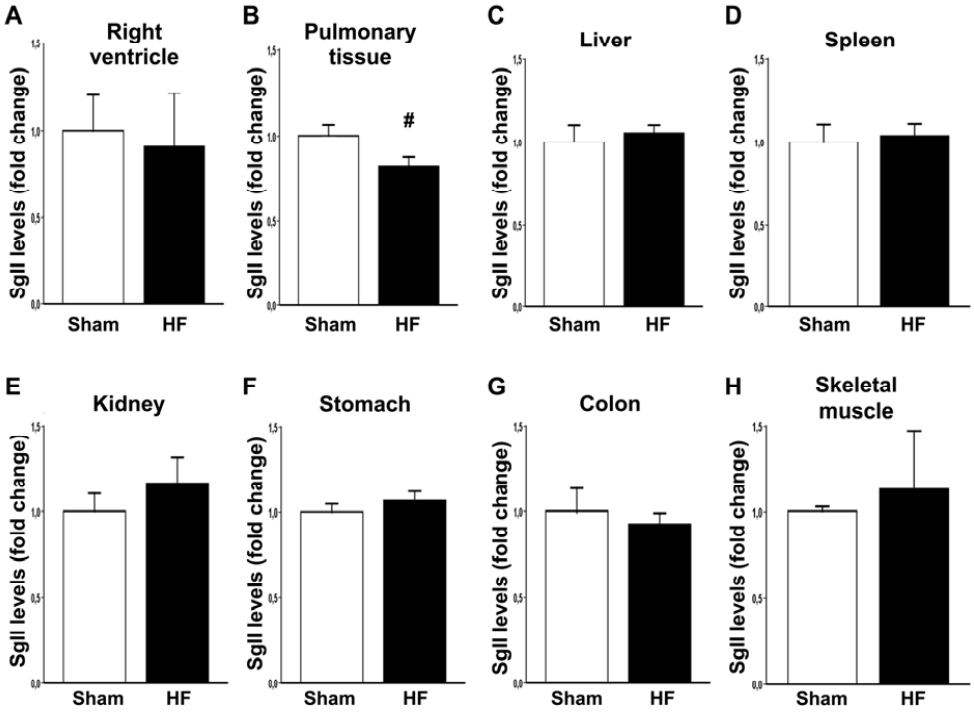

A
Right ventricle
B
Pulmonary tissue
#
C
Liver
D
Spleen
E
Kidney
F
Stomach
G
Colon
H
Skeletal muscle
SglI levels (fold change)
Sham
HF

Figure 5.

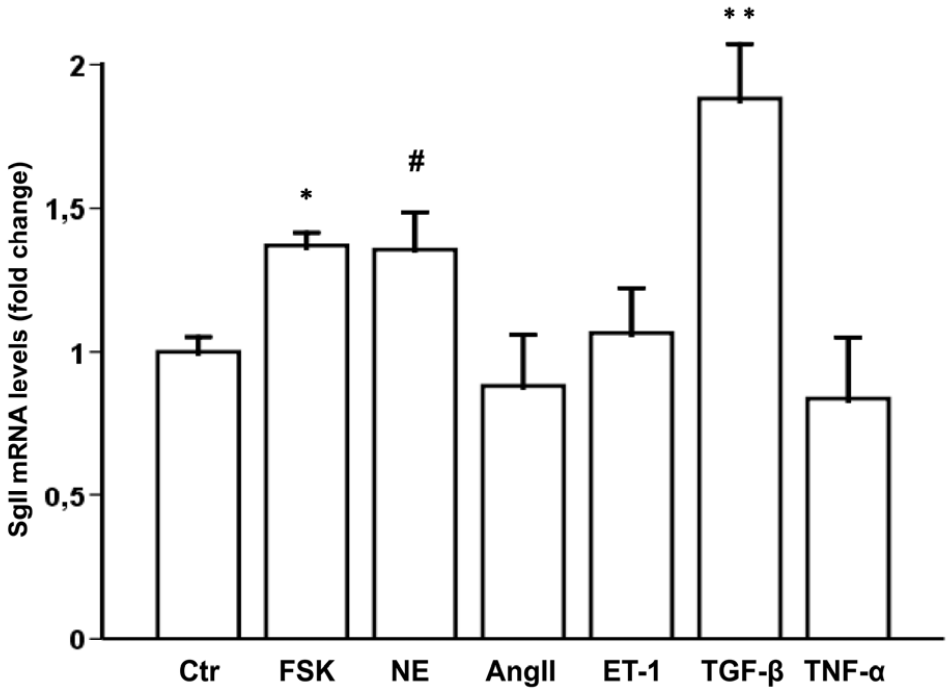

SgII mRNA levels (fold change)
2
1,5
1
0,5
0
*
#
* *
Ctr
FSK
NE
AngII
ET-1
TGF-β
TNF-α

Figure 6.

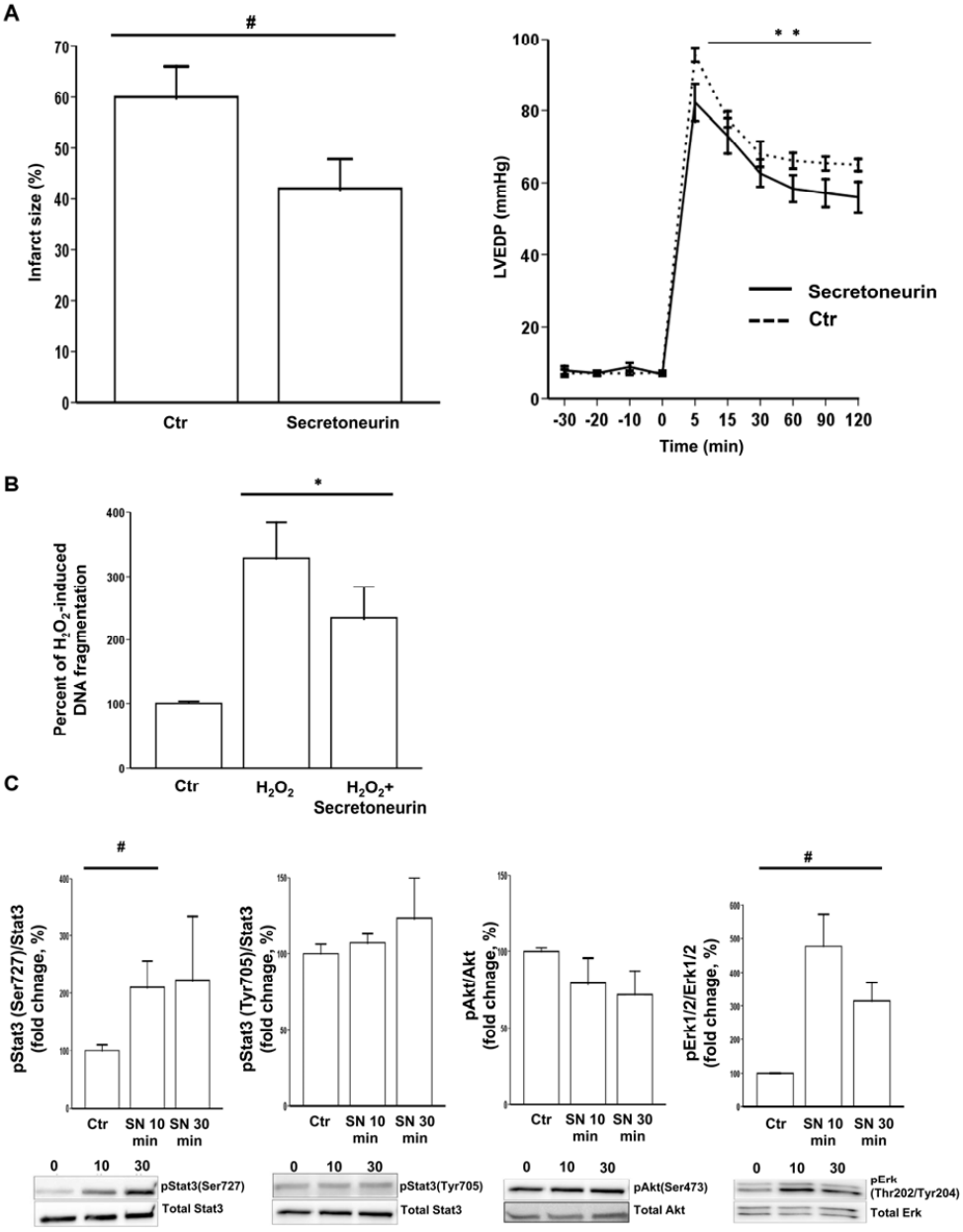
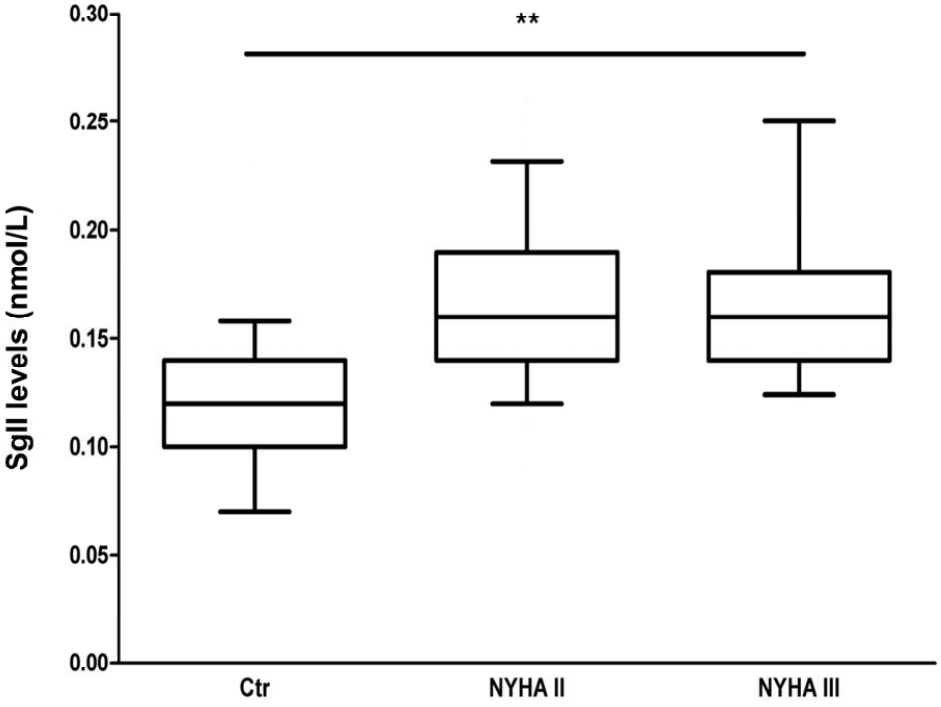

A
#
Infarct size (%)
Ctr
Secretoneurin
* *
LVEDP (mmHg)
Time (min)
Secretoneurin
Ctr
B
*
Percent of H2O2-induced DNA fragmentation
Ctr
H2O2
H2O2+ Secretoneurin
C
pStat3 (Ser727)/Stat3 (fold chnage, %)
pStat3 (Tyr705)/Stat3 (fold chnage, %)
pAkt/Akt (fold chnage, %)
pErk1/2/Erk1/2 (fold chnage, %)
Ctr
SN 10 min
SN 30 min
pStat3(Ser727)
Total Stat3
pStat3(Tyr705)
Total Stat3
pAkt(Ser473)
Total Akt
pErk (Thr202/Tyr204)
Total Erk

Figure 7.


**
0.30
0.25
0.20
0.15
0.10
0.05
0.00
SgII levels (nmol/L)
Ctr
NYHA II
NYHA III

SUPPLEMENTARY MATERIAL

to

Secretogranin II; a protein increased in the myocardium and circulation in heart failure with cardioprotective properties

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SUPPLEMENTAL METHODS

Mouse model of HF

Six week old C56BL/6 mice (Taconic, Skensved, Denmark) were used for experiments. Surgical procedures were performed as earlier described with a permanent ligation of the left main coronary artery in the heart failure (HF) group after a left-sided thoracotomy and pericardectomy [1]. Sham-operated animals (sham) were subjected to the same procedure except ligation of the coronary artery. A full echocardiographic examination was performed one week after the primary operation while animals were anesthetized breathing a gas mixture of oxygen and isoflurane supplied via a facemask. Inclusion in the HF group was based on criteria previously validated by our group as sensitive and specific for diagnosing HF: 1) Myocardial infarction >40 % of the circumference of the left ventricle (LV), 2) left atrial diameter >2.0 mm, 3) >35 % increase in lung weight compared to the sham group (e.g. lung weight >0.2 g) [2].

After sacrificing the animals, hearts and the other organs were dissected, prepared, and stored as previously described [1]. In the LV, the thin infarcted region was dissected from the hypertrophic non-infarcted region. To avoid interference of necrotic cardiomyocytes to non-infarcted LV tissue, the transformation zone (border zone) was included in the infarcted region. Tissue for RT-qPCR analysis or immunoblotting were immediately frozen in liquid nitrogen and stored at 70°C until use.

Quantitative real-time PCR (RT-qPCR)

RNA was extracted from the non-infarcted region of mice LV tissues using the SV Total RNA Isolation System (Promega Corporation, Madison, WI, USA) as previously described (20-35 mg, 9 HF mice and 8 sham) [1]. From neonatal rat cardiomyocytes, total RNA was isolated with the RNeasy mini kit (Qiagen, Hilden, Germany), RNA concentration and quality was assessed [1], and cDNA produced with the High-Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA, USA) or the iScript cDNA Synthesis Kit (Bio-Rad Laboratories Inc., Hercules, CA, USA). The results were detected on a 7900 HT Real-Time PCR System (Applied Biosystems), and the relative gene expression was determined by the standard curve method [3]. All samples were run in triplicate, RPL4 served as an internal control, and levels are presented as fold change (change from the mean in the sham group) ± SEM.

Radioimmunoassay on tissue homogenate

Frozen myocardial tissue samples were homogenized and pre-treated with SDS, and the total protein content measured as previously described [1]. SgII levels were measured by an in-house made region-specific radioimmunoassay (RIA) detecting the secretonurin part of SgII ($SgII_{154-165}$) [4]. No sample had SgII levels below the detection limit (<1 fmol/tube). All samples were measured in duplicate.

1-D gel electrophoresis and immunoblotting

We performed 1-D gel electrophoresis and immunoblotting according to standard procedures. Total protein content was measured with the micro BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA), and the lysates were denaturated for 5 min at 100°C prior to gel loading [1]. We used polyacrylamide gels (10-12 %) and the Precision Plus Protein Dual Color Standard (161-0374) as the molecular marker (Bio-Rad Laboratories Inc.). A Mini Trans-Blot Cell system (Bio-Rad Laboratories Inc.) was used to transfer proteins from gels to Hybond-P PVDF membranes (RPN303F, Amersham Biosciences Europe, Freiburg, Germany). Non-specific binding to the membrane was blocked with 5% dry milk in TBS/1% Tween for 2 h. After an overnight incubation with the primary monoclonal mouse anti-SgII antibody (diluted 1:5000, ab20246, Abcam, Cambridge, UK) at 4°C, the membranes were incubated for 1 h with a secondary goat anti-mouse IgG antibody (1030-50, Southern Biotech, Birmingham, AL, USA). Membranes were washed in between and after all incubation steps with TBS/1% Tween. The primary polyclonal anti-PC1/3 antibody was from Millipore, Billerica, MA, USA (diluted 1:500, AB10553) and the primary polyclonal anti-PC2 antibody was from Santa Cruz Biotechnology, Santa Cruz, CA, USA (diluted 1:100, sc-22891). The secondary antibody for PC1/3 was goat anti-rabbit IgG antibody (4030-05, Southern Biotech) and for PC2 a rabbit anti-goat IgG antibody (6160-05, Southern Biotech). The band at 110 kDa was measured for PC1/3 according to the manufacturer's specifications [5], while the bands at 75 and 68 kDa were assessed to determine the pro-form and active form of PC2, respectively [6].

We used a similar protocol to assess Stat3, Akt, and Erk1/2 regulation in cardiomyocytes after secretoneurin stimulation. Membranes were first incubated with phosphospesific antibodies against Stat3 (Tyr_{705} or Ser_{724}), Akt (Ser_{473}), and Erk1/2 ($_{Thr202/Tyr204}$), then stripped, before being reprobed with antibodies against total Stat3, Akt and Erk1/2 for equal protein loading (all antibodies from Cell Signaling Technologies, Beverly, MA, USA). Levels of

phosphorylated protein forms were normalized to the corresponding total level of Stat3, Akt or Erk1/2.

Immunohistochemistry

The distribution of SgII in the myocardium was examined in 3 mice with HF. Hearts were removed, fixed overnight in 4% formalin, washed in 30% ethanol and stored in 70% ethanol at 4°C before use. We used a SgII antibody binding to the C-terminal region of secretoneurin ($SgII_{172-186}$) [7] that were followed by anti-goat IgG (Vector Laboratories, Burlingame, CA, USA). The avidin-biotin-peroxidase system (Vectastain Elite kit, Vector Laboratories) was used to further amplify immunoreactivity, before sections were counter-stained with hematoxylin [1]. To investigate unspecific staining, we used non-immune rabbit serum or omitted the primary antibody.

Langendorff perfusion

Male Wistar rats (250-350 g, Scanbur AS, Nittedal, Norway) were used for these experiments. Animals were acclimatized for at least four days before any experiments were conducted. The heart temperature was kept constant during the experiment by the surrounding glass tube (inner diameter 40 mm, height 80 mm) perfused with water from the heating chamber. A fluid-filled latex balloon was inserted into the LV to measure ventricular pressures by a Powerlab system (AD Instruments Pty Ltd, Castle Hill, NSW 2154, Australia). LV end-diastolic pressure (LVEDP) was set to 5-10 mmHg and changes in LVEDP were measured. Myocardial temperature was controlled by inserting a temperature probe in the right ventricle. The hearts with LV systolic pressure ≤ 100 mmHg, coronary flow ≤ 8 or ≥ 20 mL/min, heart rate ≤ 220 beats per minute before ischemia, or irreversible arrhythmias for more than 30 min during reperfusion were excluded from the study. After 120 min of reperfusion, the hearts were cut in four slices of 1 mm and three slices of 2 mm (hearts fixed in acrylic rat brain matrix by AgnThor´s AB, Lidingö, Sweden). The 2 mm slices were freeze clamped in liquid nitrogen and stored for later analyses, while the other slices collected 5-8 mm from apex were incubated in 1% triphenyltetrazoliumchloride for 15 min at 37ºC. After incubation the slices were gently pressed between two glass plates and photographed (Nikon, Colorfix5400, Tokyo, Japan).

Cell culture experiments

Neonatal (1-3 days) Wistar rats (Taconic) were used for extraction of cardiomyocytes as previously reported [8]. Total RNA was isolated, quality assessed, and mRNA levels determined as described above. To assess the short-term effect of secretoneurin stimulation on phospho proteins, the cardiomyocytes were stimulated for 10 or 30 min with 10 μg/mL secretoneurin (NeoMPS, Strasbourg, France) or vehicle. Cells were harvested in lysis buffer (Tris pH 7.6, 5M NaCl, 0.5M EDTA, 0.1M EGTA, 1M B-gly [Sigma-Aldrich St. Louis, MO, USA] and NP-40), and the protein levels measured by Western blotting as described above.

Patients with HF and healthy control subjects

Inclusion of HF patients and healthy control subjects were performed as previously described [1]. For this study we only included stable HF patients, e.g. patients not hospitalized for worsening of HF during the last three months prior to study commencement. A transthoracic echocardiogram had been obtained within the last 18 months in 55 of the 58 patients (95%). The last 3 patients had been clinically stable during this period as evaluated by no change in severity of HF symptoms or New York Heart Association (NYHA) functional class in the last 12 months, and no hospitalization for worsening HF in the last 24 months. One of these patients had a LV ejection fraction of 20% and no evidence of reversible myocardial ischemia when evaluated by myocardial single photon emission computed tomography (SPECT) during the last year prior to study inclusion. Fifty-four patients (93%) had undergone coronary angiography for diagnostic purposes. New York Heart Association (NYHA) functional class were determined by one investigator (HR). All patients were included from Akershus University Hospital's HF outpatient clinic.

Blood samples were processed as previously described [1], and plasma B-type natriuretic peptide (BNP) levels measured by a two-step sandwich immunoassay (Architect® BNP assay, Abbott Diagnostics, Abbott Park, IL, USA). Plasma CgA levels were measured by a commercial RIA identifying $CgA_{116-439}$ (Euro-Diagnostica AB, Malmö, Sweden) and CgB levels assessed by a region-specific RIA detecting $CgB_{439-451}$ [9].

SUPPLEMENTAL REFERENCES

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(2) Finsen AV, Christensen G, Sjaastad I (2005) Echocardiographic parameters discriminating myocardial infarction with pulmonary congestion from myocardial infarction without congestion in the mouse. J Appl Physiol 98:680-689.

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(4) Stridsberg M, Eriksson B, Janson ET (2008) Measurements of secretogranins II, III, V and proconvertases 1/3 and 2 in plasma from patients with neuroendocrine tumours. Regul Pept 148:95-98.

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(6) Berman Y, Mzhavia N, Polonskaia A, Furuta M, Steiner DF, et al (2000) Defective prodynorphin processing in mice lacking prohormone convertase PC2. J Neurochem 75:1763-1770.

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(9) Stridsberg M, Eriksson B, Öberg K, Janson ET (2005) A panel of 13 region-specific radioimmunoassays for measurements of human chromogranin B. Regul Pept 125:193-199.

Supplementary Table S1. Descriptive statistics of animals

	Sham (n=21)	HF (n=27)	p
Animal weight, day 0 (g)	24.4 ± 0.4	24.4 ± 0.4	0.95
Lung weight/tibial length (g/mm)	0.079 ± 0.001	0.156 ± 0.007	<0.001
LV mass/tibial length (g/mm)	0.045 ± 0.001	0.058 ± 0.001	<0.001
RV mass/tibial length (g/mm)	0.011 ± 0.001	0.013 ± 0.001	0.01
LV CgA mRNA levels	1.0 ± 0.4	4.8 ± 1.4	0.02
LV CgB mRNA levels	1.0 ± 0.1	5.2 ± 0.7	<0.001
LV BNP mRNA levels	1.0 ± 0.1	5.8 ± 0.7	<0.001

LV indicates left ventricle; RV, right ventricle; CgA, chromogranin A; CgB, chromogranin B; and BNP, B-type natriuretic peptide. mRNA levels were investigated in a subset of animals (n=9 HF, n=8 sham) and are presented as fold change ± SEM.

Supplementary Table S2. Correlations between mRNA levels of granins and BNP in the left ventricle of heart failure and sham-operated mice

	SgII mRNA levels	
	Sham animals	**HF animals**
CgA mRNA levels	r= 0.81, p= 0.02	r= 0.68, p= 0.04
CgB mRNA levels	r= 0.10, p= 0.82	r= 0.18, p= 0.64
BNP mRNA levels	r= -0.02, p= 0.96	r= 0.38, p= 0.31

Supplementary Table S3. Correlations between circulating levels of granin proteins and BNP in patients with heart failure and healthy control subjects

	SgII levels	
	Control subjects	**HF patients**
CgA levels	r= -0.26, p= 0.27	r= 0.16, p= 0.25
CgB levels	r= -0.34, p= 0.14	r= 0.09, p= 0.49
BNP levels	r= 0.19, p= 0.43	r= 0.26, p= 0.05

Supplementary Figure. Langendorff perfusion. Hearts from male adult rats were rapidly excised and mounted on a Langendorff system. After 40 min of stabilization, hearts were subjected to 30 min of global ischemia, and then reperfused for 120 min. In the experimental group (secretoneurin group), 0.66 µg/mL secretoneurin (SN) was added to the perfusate 20 min prior to ischemia, and also used throughout the reperfusion period.